The Effect of Folic Acid Supplementation on Chemosensitivity of Human Breast Cancer Cells in an \textit{in vitro} Model

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Nutritional Sciences
University of Toronto

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Abstract

In North America, blood folate levels have dramatically increased due to mandatory folic acid (FA) fortification and widespread supplement use. Breast cancer patients report highest FA containing supplement use. 5-fluorouracil (5-FU) is the cornerstone of breast cancer chemotherapy, mechanisms of action which are based in part on interruption of folate metabolism. Concerns have been raised whether increased folate status may interfere with 5-FU chemotherapy for breast cancer. This study investigated whether FA supplementation can affect chemosensitivity of human breast cancer cells to 5-FU in 3 distinct human breast cancer cell lines. FA supplementation significantly decreased 5-FU chemosensitivity in MDA-MB 231 cells, significantly increased 5-FU chemosensitivity in HCC1937 cells and induced an inverted U shape response in MCF-7 cells. Given the high FA intake in breast cancer patients, the results from this study are concerning and future studies are warranted to clarify the effect of FA supplementation on 5-FU chemosensitivity.
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# Table of Contents

Acknowledgements ........................................................................................................... iii

Table of Contents .................................................................................................................. vi

List of Abbreviations ............................................................................................................ x

List of Tables .......................................................................................................................... xii

List of Figures ....................................................................................................................... xiii

1 Chapter 1: Introduction ..................................................................................................... 1

2 Chapter 2: Literature Review ............................................................................................. 5

  2.1 Breast Cancer ................................................................................................................. 5

  2.1.1 An overview of breast cancer ...................................................................................... 5

  2.1.2 Breast cancer carcinogenesis ..................................................................................... 6

  2.1.3 Molecular subtypes of breast cancer ......................................................................... 12

  2.1.4 Risk factors of breast cancer ...................................................................................... 14

  2.1.5 Breast cancer prevention ............................................................................................ 18

  2.1.6 Treatment for breast cancer ....................................................................................... 19

  2.1.7 5-fluorouracil based chemotherapy .......................................................................... 21

  2.1.7.1 Mechanisms of 5-fluorouracil ............................................................................... 23

  2.1.7.2 Mechanisms of acquired drug resistance to 5-FU .................................................. 25

  2.2 Folate ............................................................................................................................ 28

  2.2.1 An overview of folate ................................................................................................. 28

  2.2.1.1 Dietary sources and recommendations ................................................................. 28

  2.2.2 Chemical Structure .................................................................................................... 29

  2.2.3 Folate absorption and metabolism ........................................................................... 30

  2.2.4 Biochemical functions of folate ................................................................................ 32

  2.2.4.1 Regeneration of methionine .................................................................................. 32

  2.2.4.2 Biological methylation reactions .......................................................................... 32

  2.2.4.3 Nucleotide biosynthesis ....................................................................................... 33

  2.3 Folate and Health ........................................................................................................... 35
4.2.1 Cell lines ................................................................. 74
4.2.2 Cell culture ............................................................. 75
4.2.3 Growth curves .......................................................... 75
4.2.4 Determinants of intracellular folate concentrations .......... 76
  4.2.4.1 Folic acid standard preparation ................................ 77
  4.2.4.2 Lactobacillus rhamnosus stock preparation ................. 77
  4.2.4.3 Chicken pancreas conjugase preparation ..................... 77
  4.2.4.4 Intracellular folate determination ................................ 78
4.2.5 Drug preparation ........................................................ 79
4.2.6 In vitro chemosensitivity ............................................... 80
4.2.7 Semi-quantitative real time reverse transcriptase-PCR .......... 83
  4.2.7.1 Total RNA extraction ............................................ 83
  4.2.7.2 Synthesis of cDNA ............................................... 84
  4.2.7.3 Semi-quantitative real time RT-PCR ......................... 85
4.2.8 Statistical Analyses ..................................................... 86
4.3 Results ........................................................................... 88
  4.3.1 Growth curves .......................................................... 88
  4.3.2 Intracellular folate concentrations ................................ 90
  4.3.3 In vitro chemosensitivity to 5-FU ................................ 92
    4.3.3.1 HCC1937 chemosensitivity to 5-FU ....................... 92
    4.3.3.2 MCF-7 chemosensitivity to 5-FU ............................ 95
    4.3.3.3 MDA-MB-231 chemosensitivity to 5-FU ................. 98
  4.3.4 The effect of FA on gene expression via quantitative RT-PCR 101
  4.3.5 Summary .................................................................. 105
4.4 Discussion ....................................................................... 106
  4.4.1 Growth curves .......................................................... 106
  4.4.2 Intracellular folate concentrations varied based on FA concentrations in the medium ........................................... 110
  4.4.3 In vitro chemosensitivity to 5-FU and gene expression via quantitative RT-PCR 114
    4.4.3.1 HCC1937 cells..................................................... 114
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDR</td>
<td>Basal dietary requirement</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer Susceptibility Gene-1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast Cancer Susceptibility Gene-2</td>
</tr>
<tr>
<td>CCO</td>
<td>Cancer Care Ontario</td>
</tr>
<tr>
<td>CHMS</td>
<td>Canadian Health Measures Survey</td>
</tr>
<tr>
<td>CMF</td>
<td>Cyclophosphamide, methotrexate and 5-fluorouracil</td>
</tr>
<tr>
<td>DFE</td>
<td>Dietary folate equivalent</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolate</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DHFU</td>
<td>5,6-dihydrofluorouracil</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DPD</td>
<td>Dihydropyrimidine dehydrogenase</td>
</tr>
<tr>
<td>dTMP</td>
<td>Deoxynucleosine monophosphate</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FA</td>
<td>Folic acid</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>5FdUMP</td>
<td>5-fluoro-deoxouridine-monophosphate</td>
</tr>
<tr>
<td>5FdUTP</td>
<td>5-fluoro-deoxouridine-triphosphate</td>
</tr>
<tr>
<td>FEC</td>
<td>5-fluorouracil, epirubicin, and cyclophosphamide</td>
</tr>
<tr>
<td>FEC-D</td>
<td>5-fluorouracil, epirubicin, cyclophosphamide-docetaxal</td>
</tr>
<tr>
<td>FPGS</td>
<td>Folylpolyglutamate synthase</td>
</tr>
<tr>
<td>FR</td>
<td>Folate receptor</td>
</tr>
<tr>
<td>5FUUMP</td>
<td>5-fluoro-uridine-monophosphate</td>
</tr>
<tr>
<td>5FUTP</td>
<td>5-fluoro-uridine-triphosphate</td>
</tr>
<tr>
<td>GCP II</td>
<td>Glutamate carboxypeptidase II</td>
</tr>
<tr>
<td>GGH</td>
<td>y-glutamyl hydrolase</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HR</td>
<td>Hormone receptor</td>
</tr>
<tr>
<td>LV</td>
<td>Leucovorin (also known as folinic acid or 5-formylTHF)</td>
</tr>
<tr>
<td>MAT</td>
<td>Methionine adenosyltransferase</td>
</tr>
<tr>
<td>MNU</td>
<td>N-Methyl-N-nitrosourea</td>
</tr>
<tr>
<td>MRP</td>
<td>Multi-drug resistance pump</td>
</tr>
<tr>
<td>MS</td>
<td>Methionine synthase</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>NTD</td>
<td>Neural tube defect</td>
</tr>
<tr>
<td>PABA</td>
<td>Para-aminobenzoic acid</td>
</tr>
<tr>
<td>PCFT</td>
<td>Proton-coupled folate transporter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized clinical trial</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended dietary allowance</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced-folate carrier</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>UMFA</td>
<td>Unmetabolized folic acid</td>
</tr>
<tr>
<td>UL</td>
<td>Upper limit</td>
</tr>
</tbody>
</table>
List of Tables

Table 2.1.4.1. Factors that modify breast cancer risk in premenopausal women. .................. 15
Table 2.1.4.2. Factors that modify breast cancer risk in postmenopausal women................. 16
Table 2.1.7.2. List of selected ABC transporter proteins linked to multidrug resistance. ........ 26
Table 2.4.2. Dual modulatory role of folate in carcinogenesis. ........................................ 57
Table 2.4.3.1. Summary of studies that have directly examined the effect of FA deficiency on 5-FU chemosensitivity. ......................................................................................... 63
Table 2.4.3.2. Summary of studies that have directly examined the effect of FA supplementation on 5-FU chemosensitivity. ................................................................. 64
Table 4.2.1. Phenotypic and molecular characteristics of HCC1937, MCF-7 and MDA-MB-231 human breast cancer cell lines. ................................................................. 75
Table 4.2.4.1. Composition of 0.1 M KPO₄ buffer and folic acid media for folate assay. .......... 79
Table 4.2.5. Preparation of 5-FU for in vitro chemosensitivity. ................................................ 80
Table 4.2.7.3. Primer sequences used for quantitative RT-PCR. ........................................... 86
Table 4.3.5. Summary of growth curve, intracellular folate concentration, chemosensitivity and relative gene expression................................................................. 106
List of Figures

Figure 2.1.2. Molecular phases of breast carcinogenesis: initiation, promotion, progression, and metastasis. ............................................................................................................................................. 6

Figure 2.1.3. The prevalence, receptor expression, histologic grade, prognosis and response to treatment of different molecular subtypes of breast cancer. .......................................................... 13

Figure 2.1.6.1. Treatment algorithm depicted for early stage breast cancer depending on menopausal status. ................................................................................................................................. 20

Figure 2.1.6.2. Treatment algorithm depicted for invasive stage IV breast cancer. ............... 21

Figure 2.1.7.1 Metabolism of 5-fluorouracil. ........................................................................... 23

Figure 2.2.2: Chemical structure of [A] folic acid and [B] folate. ........................................... 30

Figure 2.2.3 Folate absorption, metabolism, and biochemical functions ............................... 34

Figure 2.4.1.1 Relative risks (RRs; in prospective studies) or odds ratios (ORs; in case–control studies) of breast cancer comparing the highest with the lowest dietary folate intake categories................................................................................................................................. 52

Figure 4.2.6. Schematic depicting the five day in vitro chemosensitivity assay using Sulforhodamine B (SRB) protein dye. .......................................................................................................... 82

Figure 4.3.1. Fifteen day growth curve of (A) HCC1937, (B) MCF-7 and (C) MDA-MB-231 cell lines cultured in 5, 50, 500 and 1000 nM FA RPMI 1640 medium. ......................................................... 89

Figure 4.3.2. Intracellular folate concentrations of HCC1937, MCF-7 and MDA-MB-231 cells cultured in 5, 50, 500 and 1000 nM FA medium concentration at days 0, 5, 10 and 15. .......... 91

Figure. 4.3.3.1.1 Predicted percent survival for HCC1937 cells based on final regression model: percent survival = (date) (FA) (LV) (log 5-FU) (FA*LV) (log 5-FU*LV), where * indicates an interaction. ................................................................................................................................. 92

Figure. 4.3.3.1.2 Comparison between predicted percent survival for HCC1937 cells based on final regression model treated with 5-FU alone or 5-FU with the addition of LV among cells grown in different FA medium concentrations......................................................... 93

Figure. 4.3.3.2.1 Predicted percent survival for MCF-7 cells based on final regression model: percent survival = (date) (FA) (LV) (log 5-FU) (FA*LV) (log 5-FU*FA) (log 5-FU*LV), where * indicates an interaction. ................................................................................................................................. 95
Figure 4.3.3.2.2 Comparison between predicted percent survival for MCF-7 cells based on final regression model treated with 5-FU alone or 5-FU with the addition of LV among cells grown in different FA medium concentrations........................................96

Figure 4.3.3.3.1 Predicted percent survival for MDA-MB-231 cells based on final regression model: percent survival = (date) (FA) (LV) (log 5-FU) (FA*LV) (FA*log 5-FU), where * indicates an interaction. ..........................................................................................................................98

Figure 4.3.3.3.2 Comparison between predicted percent survival for MDA-MB-231 cells based on final regression model treated with 5-FU alone or 5-FU with the addition of LV among cells grown in different FA medium concentrations.................................................................................99

Figure 4.3.4.1. Fold change in TS expression on day 5 relative to day 0 in MDA-MB-231, MCF-7 and HCC1937 cell lines cultured at 5, 50, 500 and 1000 nM FA medium. ...............................................102

Figure 4.3.4.2. Fold change in MTHFR expression on day 5 relative to day 0 in MDA-MB-231, MCF-7 and HCC1937 cell lines cultured at 5, 50, 500 and 1000 nM FA medium. .........................102

Figure 4.3.4.3. Fold change in DHFR expression on day 5 relative to day 0 in MDA-MB-231, MCF-7 and HCC1937 cell lines cultured at 5, 50, 500 and 1000 nM FA medium. .........................103

Figure 4.3.4.4. Fold change in MRP1 (panel A) and MRP5 (panel B) expression on day 5 relative to day 0 in MDA-MB-231, MCF-7 and HCC1937 cell lines cultured at 5, 50, 500 and 1000 nM FA medium. ...........................................................................................................................................104

Figure 4.3.4.5. Fold change in MRP8 expression on day 5 relative to day 0 in MCF-7 cells cultured at 5, 50, 500 and 1000 nM FA medium. ........................................................................................................105
Chapter 1: Introduction

In the Western World, breast cancer continues to be the most commonly diagnosed cancer in women [1, 2]. In Canada it is the leading cause of death in women, accounting for 14% of all cancer deaths [3]. Due to improvements in screening, leading to early detection and improvement in treatment modalities, there has been a steady decline in age-standardized mortality rates to 43% over the past two decades in Canada [3]. Despite this trend in increasing survival, patients presenting with more aggressive subtypes of breast cancer that cannot be treated with targeted therapies only have a 5 year survival rate of 23% [3]. These patients have very poor prognosis since these cancers generally rapidly metastasize. Among several systemic combination chemotherapy formulations used in the treatment of breast cancers, 5-Fluorouracil (5-FU) is a common active ingredient [4]. Importantly, according to the National Comprehensive Cancer Network (NCCN) and Cancer Care Ontario (CCO) guidelines, the standard adjuvant chemotherapy formulation for breast cancers is FEC-D (5-FU, epirubicin, cyclophosphamide-docetaxal) and it is the primary line of treatment for aggressive subtypes of breast cancer [4-7].

5-FU is a pyrimidine antagonist prodrug that must be converted intracellularly into its three active cytotoxic metabolites [8]. The main cytotoxicity of 5-FU in cancer cells is due to the formation of an inhibitory ternary complex composed by its cytotoxic metabolite, 5-fluorodeoxyuridine-monophosphate (5FdUMP), thymidylate synthase (TS) and 5,10-methyleneTHF, thereby inhibiting TS and suppressing DNA synthesis [8]. Clinically, 5-FU is administered with leucovorin (LV; 5-formylTHF), since LV, a precursor of 5,10-methyleneTHF, increases
intracellular concentrations of 5,10-methyleneTHF, thereby enhancing the formation and stabilization of the inhibitory 5,10-methyleneTHF-TS-5FdUMP ternary complex [8]. Importantly, TS and 5,10-methyleneTHF are an important enzyme and metabolite in DNA synthesis and folate metabolic pathways, respectively.

Folate, a water soluble B vitamin (vitamin B9), and its synthetic form, folic acid (FA), are important for human health as they mediate one-carbon transfer reactions involved in nucleotide biosynthesis and biological methylation reactions [9]. In this role, folate plays important functions in DNA synthesis, integrity and repair as well as DNA methylation critical for regulation of gene expression [9]. As such, both folate deficiency and excess have been associated with a variety of adverse health outcomes [10-12]. A convincing body of evidence on periconceptional FA supplementation reducing risk of neural tube defects (NTDs) led to the implementation of mandatory FA fortification in 53 countries, including the United States (U.S.) and Canada [13-15]. In addition, approximately 30-40 % of the North American population consumes FA containing supplements [16-19]. At present, FA fortification of the food supply combined with widespread supplement use, usually containing 400 µg of FA, has resulted in high intakes and blood levels of folate and FA in North America [20, 21]. In Canada, based on data from the most recent Canadian Health Measures Survey (CHMS), >40% of the population had red blood cell (RBC) folate concentrations above the level generally considered to be high (defined as levels above the 97th percentile of RBC folate concentrations from the National Health and Nutrition Examination Survey (NHANES) 1999-2004 at 1360 nmol/L) [21]. Folate and FA intake appears to be even higher in cancer patients and survivors as 14-32 % of cancer
patients initiate supplement use after diagnosis [22]. Of all cancer patients, breast cancer patients were found to have the highest prevalence of multivitamin use at 57-62% [22].

High dietary intake of folate and FA and prevalent supplement use in breast cancer patients is concerning given emerging evidence on the role of folate in breast cancer. Even though the evidence concerning the association of folate status and breast cancer risk is inconclusive, recent epidemiologic studies have reported increased risk of breast cancer with high folate status [23-28]. Evidence from in vivo studies is also conflicting but a recent animal study has reported that FA supplementation between 2.5x and 5x the basal dietary requirement (BDR) in rats significantly increased the progression of 7,12-dimethylbenz[a]anthracene (DMBA) induced mammary tumors [29]. Evidence from animal studies and clinical trials in other cancer sites (e.g., colorectal cancer, prostate cancer) have suggested a dual modulatory role of folate in cancer development and progression [10, 30, 31]. In normal cells, folate deficiency increases, whereas modest levels of FA supplementation suppresses, the risk of neoplastic transformation [10]. In contrast, folate deficiency inhibits, whereas FA supplementation promotes, the progression of (pre)neoplastic lesions [10].

An emerging body of evidence suggests that folate status can influence the sensitivity of cancer cells to 5-FU and anti-folate-based chemotherapy. Several studies have reported that folate deficiency increases chemosensitivity to 5-FU [32-34]. However, conflicting evidence exists for the effect of FA supplementation on sensitivity of cancer cells to 5-FU. Two in vivo studies suggest that FA supplementation can increase sensitivity of breast cancer cells to 5-FU [35, 36]. A recent study, however, demonstrated that FA supplementation can promote the growth of human colon cancer xenografts in nude mice receiving 5-FU treatment and negate
the cytotoxic effect of 5-FU [37]. These observations suggest that FA supplementation decreases the efficacy of 5-FU treatment. At present, whether or not FA supplementation can enhance or interfere with the cytotoxic effect of 5-FU-based chemotherapy in breast cancer is largely unknown.

Considering the high incidence of breast cancer in women worldwide, the prevalent use of supplements by breast cancer patients, and the common use of 5-FU in breast cancer treatment, whether or not FA supplementation may decrease chemosensitivity of breast cancer cells to 5-FU is an important clinical and public health issue. There is a paucity of studies examining the effect of FA supplementation on chemosensitivity of breast cancer cells to 5-FU and to the best of our knowledge, there are no published data from in vitro studies. It is important to determine optimal FA supplemental levels in breast cancer patients during treatment with 5-FU-based chemotherapy to reduce side effects associated with folate insufficiency, which may result from chemotherapy and/or nutritional depletion, while maximizing the efficacy of the treatment. Given these considerations, the goal of my Master’s thesis was to investigate the role of FA supplementation in modulating sensitivity of breast cancer cells to 5-FU in an in vitro model of breast cancer and to elucidate potential underlying mechanisms.


2 Chapter 2: Literature Review

2.1 Breast Cancer

2.1.1 An overview of breast cancer

Cancer continues to be the leading cause of death, responsible for 30% of all deaths [1]. In the Western World, breast cancer continues to be the most commonly diagnosed cancer in women, accounting for 23% of the total cancer cases and 14% of the cancer deaths [1, 2]. In Canada, according to the Canadian Cancer Society Cancer Statistics latest report, breast cancer is the most common cancer among Canadian women, excluding non-melanoma skin cancers, and the leading cause of death from cancer in women [3]. In 2016, breast cancer is estimated to account for 26% of all new cancer cases diagnosed in women and 14% of all cancer deaths [3]. Since breast cancer mortality reached its all-time peak in 1986, the age-standardized mortality rates have steadily declined to 43% and overall 5-year survival has increased to 88% in Canada [3]. This has been attributed to improvements in screening leading to early detection and more targeted treatment options. Despite this trend in increasing survival, in the Western world breast cancer still remains the second-leading cause of cancer-related death in women [1]. In Canada, patients presenting with metastatic breast cancer, 5% of breast cancer patients diagnosed, have a 5-year survival rate of only 23% [1]. Due to high morbidity and health costs associated with breast cancer, there is an urgent need for further research to improve treatment options for breast cancer patients who present with an aggressive subtype.
2.1.2 Breast cancer carcinogenesis

Similar to other cancers, breast cancer is characterized by uncontrolled proliferation, specifically of breast epithelial cells, the loss of cellular differentiation of these cells and the ability to migrate to other parts of the body and establish metastases [38]. As outlined in Figure 2.1.2, this process can be classified into three phases: initiation, promotion and progression/metastasis [38].

Figure 2.1.2. Molecular phases of breast carcinogenesis: initiation, promotion, progression, and metastasis. Image adapted and reprinted by permission from the publisher (John Wiley and Sons Ltd) [39].

In the initiation stage, an epithelial cell lining the duct or lobule of the mammary gland acquires irreversible DNA damage either due to a spontaneous genetic mutation or environmental factors such as radiation, and escapes cellular repair mechanisms. These spontaneous mutations arise in all healthy individuals, but these cells harboring the aberrant mutation will either stay dormant or undergo apoptosis. When an initiated cell escapes apoptosis and additionally acquires a mutation that confers a growth or survival advantage, clonal expansion occurs which characterizes the promotion stage of carcinogenesis. During clonal expansion, the initiated cell proliferates uncontrollably, escapes DNA repair mechanisms and passes on its mutations to a cluster of new daughter cells. Depending on the site of origin,
these clusters result in localized breast cancer called ductal or lobular carcinoma *in situ*. If the cancer is caught at this early stage through screening, prognosis is favorable. However, if the cancer is undetected and these initiated cells continue to divide, new genetic and epigenetic mutations are acquired that will eventually confer the means for these cells to escape the *in situ* mass. In order to achieve this, proliferating breast cancer cells secrete signals to promote angiogenesis. This allows for subsequent invasion of the cancer cells into small blood vessel walls by producing proteolytic enzymes to allow for the cancerous cells’ penetration of the blood vessel walls and entry into the general circulation [40]. These cells travel in the circulation and if they are successful in evading the host’s immune system travel to distant organs thus entering the final stage of carcinogenesis: progression/metastasis. During this process many cancer cells die, but if some survive and reach the sinusoids of the bone marrow cavity or the capillaries of the lung and liver, these cells are in a highly favourable position to establish metastases [41]. Therefore, once breast cancer reaches the last stage of carcinogenesis, it usually metastasizes to the bone, lung and liver [42].

Breast cancer results from the stepwise genetic and/or epigenetic alterations of normal cells. Over the past decade, great advances were made in understanding specific genes, proteins and biological pathways associated with breast cancer. Numerous oncogenes have been characterized to date, but relatively few are crucial in the progression of breast cancer. The most well characterized oncogene in breast cancer is the human epithelial receptor 2 (*HER-2*) gene, located on chromosome 17q, responsible for encoding a 185 kDa transmembrane tyrosine kinase growth receptor [43]. *HER-2* becomes activated upon binding to specific ligands, or if the receptor density on the cell membrane is high enough, then
autodimerization can result, followed by receptor autophosphorylation, which leads to multiple transduction cascades acting through a variety of pathways, including the mitogen-activated protein (MAP) kinase and 3-kinase (PI3K)/Akt pathways, which lead to increased proliferation, angiogenesis, altered cell-cell interactions, increased cell motility, metastases, and resistance to apoptosis [44]. Since HER-2 is overexpressed in 20-30% of invasive breast cancer and is associated with a more aggressive phenotype, a lot of research has focused on exploiting it for diagnostic and clinical applications [45]. The epidermal growth factor receptor (EGFR, also known as HER-1), is also relevant to a lesser extent in breast cancer [46]. Both EGFR and HER-2 mediate the activation of downstream signal transduction pathways resulting in the activation of gatekeeper kinases in several pathways, such as Ras in the PI3K/Akt and MAP kinase pathways.

Proliferation is also closely regulated by cyclin-dependent kinases (CDKs), which upon activation promote the phosphorylation of other proteins, such as retinoblastoma protein (pRb), a primary gatekeeper that allows a cell to pass from a resting state, G0, into active cycling and mitosis [47]. CDKs in turn are positively regulated by cyclins and inhibited by cyclin-dependent kinase inhibitors (CKIs) [47]. Expression levels of cyclin D1 and cyclin E vary throughout the cell cycle, and both molecules play a critical role in signaling for progression from G1 to S phase [47]. Both cyclin D1 and E amplification play an important role in breast cancer carcinogenesis, although cyclin E to a lesser extent. Cyclin D1 overexpression and amplification has been observed in 40-50% and 10-20% of invasive cancers, respectively [48]. Upon cyclin 1D binding CDK, pRb becomes phosphorylated, transcriptional factor E2F is released, resulting in the upregulation of proteins involved in DNA synthesis and increased
proliferation [48]. In contrast, cases of cyclin E amplification are rare and overexpression has been observed in 20-30% of breast cancers [49]. Unlike cyclin D1, higher levels of cyclin E, results in increased proliferation due to hyperphosphorylation of pRb or the induction of cells in the S phase independent of pRb [50]. Consequently, high cyclin E expression is associated with marked dysregulation in proliferation and worse prognosis compared to cyclin D1 overexpression [49, 50]. Finally, another cell-cycle regulatory protein c-myc, a nuclear phosphoprotein that regulates cellular proliferation, differentiation, and apoptosis, is amplified and overexpressed in 15-25% of breast tumors [51]. However, there is controversy whether or not changes in Myc expression are enough to drive breast carcinogenesis. In most cases, carcinogenesis is driven by the synergistic effects of aberrant oncogene and tumor suppressor gene expression.

Tumor suppressor genes are negative regulators of growth and are protective against the development of malignancy. Furthermore, loss of function of these genes results in cancer. The most well studied tumor suppressor gene in breast cancer is p53, found in 20-30% of breast tumors [52]. The p53 gene located on chromosome 17p codes a 53-kDa protein that upon activation due to DNA damage, induces the transcription of a number of genes, including the CKI p21, resulting in the arrest of the cell cycle in the G1 or G2/M phase, allowing for DNA repair before mitosis or triggering apoptosis [53]. Another tumor suppressor, p27, is a CKI that functions as a negative regulator of cell cycle, by binding to a variety of cyclin/CDK complexes resulting in arrest in G1 phase [54]. However, it is observed in less than 1% of breast tumors [55].
Finally, two common tumor suppressor genes associated with hereditary breast cancer, breast cancer susceptibility genes 1 or 2 (BRCA1 or BRCA2) play an important part in carcinogenesis. BRCA1 mutations have been estimated to account for slightly more than 5% of all breast cancer cases occurring in women under the age of 40, and for over 90% of cases in families with a history of four or more cases of breast cancer and more than one case of ovarian cancer [56, 57]. BRCA1 encodes a protein containing a protein binding domain at the amino terminus [58] that interacts with BRCA-associated ring domain (BARD1) and repeats in the carboxy terminus commonly observed in DNA repair enzymes. Upon DNA damage, BRCA1, along with BARD1 and Rad81 locate the damaged area along the DNA and repair double-stranded DNA breaks [59]. BRCA2 also participates in homologous recombination, and the lifetime risk for breast cancer is roughly the same for women with BRCA1 and BRCA2 mutations [57].

Recently, there is a growing body of evidence that supports the cancer stem cell hypothesis in breast cancer. In this model, malignant tumors are initiated and maintained by a group of cancer stem cells, which exhibit self-renewal properties and can give rise to differentiated cells alike [60]. The first mammary stem cells were isolated by Al-Hajj et al from human breast carcinoma [61]. The breast carcinomas were fractioned based on CD44 and CD24 expression, and fractioned samples were implanted into immunodeficient mice. Interestingly, only the CD44+ CD24- fraction contained tumor-initiating activity, whereas 100-fold more cells from the CD44+ CD24+ or CD44- fractions did not result in tumor formation [61]. Al-Hajj and colleagues were able to demonstrate self-renewal by showing engrafted tumors could be serially transplanted. Despite the compelling evidence provided by this study,
the differences between normal and malignant breast cancer stem cells still remain to be elucidated.

Cancer is characterized by aberrant DNA methylation, resulting in global hypomethylation that may contribute to the activation of oncogenes, chromosomal instability and loss of heterozygosity [62]. On the other hand, another hallmark of cancer is site-specific hypermethylation, mostly in the cytosine-guanine dinucleotide (CpG) sequences in the promoter region, which may result in the silencing of tumor suppressor and mismatch genes and other critical cancer-related genes [63, 64]. Currently, cancer stem cells are thought to arise by disrupting DNA methylation patterns in order to inhibit differentiation and maintain self-renewal [65, 66]. Since folate is involved in biological methylation reactions [9], the role of folate in breast cancer stem cell development needs to be examined.

There have been no studies to date that have directly examined the effect of FA on breast cancer stem cell development. However, in 2015 Farias et al examined the effect of FA exposure on DNA methylation and colonosphere formation (floating spheroids formed by colon cancer stem cells) in human colorectal cancer cell lines [67]. Farias and colleagues demonstrated that cells grown without adequate FA had significantly impaired colonosphere forming ability [67]. Overall, increasing FA supplementation resulted in increased DNMT1, decreased DNMT3a, and no significant effect on DNMT3b protein expression. Additionally, an inverse dose response relationship was also observed between genomic DNA methyl-cytosine content and FA supplementation [67]. Since these results demonstrate a link between supplemental FA and colorectal cancer stem cell phenotype and development, the underlying mechanism may also cause a similar effect in breast cancer development.
Given the postulated mechanism of the cancer stem cell theory, curative treatment options require the elimination of the cancer stem cell population. Chemotherapy and radiation treatments can only inhibit tumor cell proliferation if tumor cells are actively dividing, but miss quiescent cancer stem cells, resulting in recurrence and drug resistance [68]. There are no studies to date that have examined the effect of 5-FU treatment on breast cancer stem cells; however CD133+ stem cancer cells have been shown to survive treatment with 5-FU in metastatic melanoma [69]. Since CD133 is also overexpressed by breast cancer stem cells, this finding is of concern. Todaro and colleagues also demonstrated that CD133+ cancer stem cells from colon carcinomas use IL-4 to prevent apoptosis and IL-4 antagonists increased chemosensitivity to 5-FU [70]. Another mechanism by which cancer stem cells can be resistant to 5-FU treatment is the overexpression of ABC transporters that are responsible for 5-FU efflux. ABC transporters are overexpressed in both normal and cancer stem cells, likely to protect against intracellular toxins [71]. Given the prevalent therapeutic resistance to 5-FU in cancer stem cells, further studies are needed to elucidate mechanisms of drug resistance in order to improve patient survival rates.

2.1.3 Molecular subtypes of breast cancer

In order to enhance treatment options, the disease etiology needs to be understood better. Traditionally, there have been three molecular subtypes of breast cancer, depending on the status of three critical receptors. The most common subtype of breast cancer accounting for 60% of cases is the hormone receptor (HR) positive subtype, where the estrogen receptor (ER) and/or progesterone receptor (PR) are overexpressed [72]. ER and PR are critical hormone receptors that regulate cell growth through transcriptional regulation of genes involved in cell
cycle regulation and through cross-talk with growth factor signaling pathways [73]. The second subtype is the HER-2 positive subtype, accounting for 20% of cases, where the HER-2 is overexpressed, and low levels of ER and PR are expressed in breast tissue [74]. HER2 is a tyrosine kinase receptor that signals through the PI3K-Akt and MAPK pathways that are pro-proliferative and anti-apoptotic [75]. The final subtype is called basal-like and accounts for the remaining 20% of cases. Of the basal-like, 85% of cases are classified triple negative breast cancer (TNBC) and are characterized by negative ER, PR and HER-2/neu status and high expression of basal epithelial genes [76]. Since the advent of microarray technology, as illustrated in Figure 2.1.3, the HR+ subtype has been further subdivided into molecular subtypes as follows: Luminal A (ER+/PR+/HER2-), Luminal B (ER+/PR+/HER2+) [75, 77].

![Molecular subtypes table and diagram]

**Figure 2.1.3.** The prevalence, receptor expression, histologic grade, prognosis and response to treatment of different molecular subtypes of breast cancer. Image adapted and reprinted by permission from the McMaster Pathophysiology Review, www.pathophys.org [78].
More recently, the St. Gallen Consensus panel agreed to add Ki-67 as a marker for proliferation. Therefore, the most recent subtype classification is as follows: Luminal A (ER+/PR+/HER2-/Ki-67 low), Luminal B (ER+/PR+/HER2+/Ki-67 high), HER2 overexpressing (ER + or -/PR+ or -/HER2+), basal (ER-/PR-/HER2-) and normal breast-like [79]. Currently, much research is conducted on other proteins such as p53 [80], PTEN [81] and caveolin-1 [82] to develop better therapeutic targets for certain subtypes of breast cancer. The hope is that molecular profiling will aid in more accurate diagnosis, prognosis, and better treatment.

Targeted pharmacotherapeutics have already led to major improvements in the care of more aggressive subtypes of breast cancer [83]. Furthermore, a better characterization and identification of molecular targets may lead to potentially more effective prevention strategies such as dietary intervention and chemopreventive agents.

2.1.4 Risk factors of breast cancer

Since the majority of breast cancer cases are hormone related, factors that modify breast cancer risk need to be considered separately according to the menopausal status. In premenopausal women, as illustrated in table 2.1.4.1, there is convincing evidence that lactation decreases, while alcohol increases, the risk. There is probable evidence that body fatness decreases the risk and adult attained height, as a marker for factors affecting growth, and greater birth weight increase the risk in premenopausal women. However, evidence on physical activity decreasing premenopausal breast cancer risk is limited [84].
Table 2.1.4.1. Factors that modify breast cancer risk in premenopausal women. This material has been reproduced from the World Cancer Research Fund International Continuous Update Project (CUP) www.wcrf.org [84].

In postmenopausal women (Table 2.1.4.2), lactation and alcohol consumption pose the same risk as in premenopausal women. In addition there is convincing evidence that adult attained height as a marker for genetic, environmental, hormonal and nutritional factors affecting growth, from conception to completion of linear growth, increases risk [84]. Unlike in premenopause, it is probable that physical activity decreases risk, and abdominal fatness and adult weight gain are probable factors that increase risk post menopause [84].
Table 2.1.4.2. Factors that modify breast cancer risk in postmenopausal women. This material has been reproduced from the World Cancer Research Fund International Continuous Update Project (CUP) www.wcrf.org [84].

There is inconclusive data on nutritional factors such as grains, dietary fibre, fruits and vegetables, soya products, meat, total fat and fatty acid composition affecting breast cancer risk [84]. However, in a recent systematic review and meta-analysis, Aune and colleagues found that blood concentrations of carotenoids were more strongly associated with reduced breast cancer risk than carotenoids assessed by dietary questionnaires [85]. Therefore, in the future using only blood concentrations may clarify inconsistencies observed in carotenoid studies. Another recent systematic review and meta-analysis of fifteen prospective studies, reported high intake of fruits, and fruits and vegetables combined, but not vegetables, is associated with a weak reduction in breast cancer risk [86]. Dietary fibre has also been extensively studied in breast cancer yielding conflicting results. A recent meta-analysis of prospective studies found an inverse association between dietary fiber intake and breast
cancer risk [87]. Moving forward, the next WCRF report may include carotenoids, fruit and vegetable intake combined, and dietary fiber in the limited-suggestive category for decreasing breast cancer risk. Furthermore, regarding dietary intervention, consuming a healthy diet that is protective against weight gain in adulthood decreases breast cancer risk postmenopause [2].

It is well established that lifetime exposure to estrogen, influenced by early menarche, late menopause, nulliparity and late (over 30) first pregnancy all increase the risk of breast cancer [2]. The converse of the aforementioned factors all decrease the risk of breast cancer. These lifetime exposure factors result in age acting as a marker for risk, with breast cancer risk doubling every 10 years until menopause [88]. It is important to note that the age at which development of breasts occur and that of menopause are both closely influenced by nutrition, since energy rich diets promote earlier puberty and late menopause, and energy deficient diets delay puberty and advance menopause [2].

Medication containing hormones, such as hormone replacement therapy and oral contraceptives containing both estrogen and progesterone increase breast cancer risk; the increased risk decreases after cessation of both treatments [2]. Environmental factors, such as ionising radiation exposure and geographic location are also well-established risk factors. Exposure from medical treatments such as x-rays, particularly during puberty and even at low doses, increases the risk [2]. Geographic location is also important; studies on migrant groups indicate that migrants assume the breast cancer rate of their new country by the second generation [88].
Hereditary breast cancers have lower incidence, accounting for approximately 4 to 9% of breast cancer cases and are usually caused by the inheritance of a deleterious mutation in one of the two breast cancer susceptibility genes 1 or 2, (BRCA1 or BRCA2). These women have a lifetime risk of breast cancer between 45% and 87% [57]. The BRCA1-associated breast cancers typically have an aggressive phenotype common to TNBC [89] while the BRCA2-associated breast cancers resemble sporadic subtypes. Although family history and genetic susceptibility are strong predictors of breast cancer, the World Cancer Research Fund estimates 40% of breast cancer cases are preventable through nutrition, food and physical activity [2]. Therefore, nutrition plays a critical role in breast cancer prevention.

2.1.5 Breast cancer prevention

The main form of risk management practiced today is mammography screening and chemoprevention using tamoxifen and aromatase inhibitors in women at risk. Curtailing alcohol consumption, increasing physical activity and dietary interventions promoting weight loss may also be recommended, in case of weight gain by more than 10-20 kg from age 18 [88]. Predictive genetic testing is available for the high risk potential BRCA mutation carriers, but the only prevention offered is prophylactic mastectomy and/or chemoprevention with tamoxifen [90]. Tamoxifen is a synthetic anti-estrogenic analog that competitively blocks estrogen receptors, and inhibits the proliferation of ER+ tumors. However, treatment with tamoxifen is associated with increased risk of endometrial cancer, uterine sarcoma, arterial and venous thromboembolism [91]. Since both mastectomy and tamoxifen treatment uptake is low among women at risk, more research needs to be done to elucidate more non-invasive and modifiable options such as dietary and lifestyle strategies that may reduce risk.
2.1.6 Treatment for breast cancer

Treatment options for breast cancer patients are outlined in the NCCN and CCO guidelines and differ considerably based on the stage, grade and molecular subtype of the tumor [4, 7]. When determining the course of local and systemic treatments, patient characteristics (e.g., age, menopausal status, comorbidities), cancer characteristics (e.g., stage, metastasis, receptor status, node status, histology reports) and the preferences of the patient must be taken into consideration [4, 7].

As shown in Figures 2.1.6.1 and 2.1.6.2, generally, breast cancer patients will undergo local treatments first such as breast conserving surgery (BCS) or mastectomy, with extensive lymph node dissection and/or radiation therapy [4, 7]. Depending on the grade and stage of the cancer, patients will then receive pre/post adjuvant single agent or combination chemoradiation or chemotherapy. Patients will be further considered to receive targeted systemic therapy in conjunction with chemotherapy and radiation therapy [4, 7].

Patients with the HR+ subtype can receive targeted endocrine therapy, such as aromatase inhibitors and selective estrogen-receptor response modulators [73]. Similarly, HER-2 overexpressing patients usually receive anti-HER-2 targeted therapy such as Herceptin in addition to chemoradiation after surgery [4, 7]. Unlike the other two subtypes, TNBC, with an aggressive phenotype and worse prognosis, does not have targeted treatments and as such can only be targeted with cytotoxic chemotherapy and radiation therapy to control micro-metastatic disease, thereby reducing the recurrence rate and increasing overall survival [4, 7].
Figure 2.1.6.1. Treatment algorithm depicted for early stage breast cancer depending on menopausal status. All patients diagnosed with ductal carcinoma in situ (DCIS) or stage I, II, or III pre or postmenopausal breast cancer will undergo local treatments first such as breast conserving surgery or mastectomy, with extensive lymph node dissection and/or radiation therapy. Depending on the grade and stage of the cancer, patients will then receive pre/post adjuvant single agent or combination chemotherapy. Patients will be further considered to receive targeted systemic therapy in conjunction with chemotherapy and radiation therapy.
Figure 2.1.6.2. Treatment algorithm depicted for invasive stage IV breast cancer. Treatment of patients with the HR+ subtype depends on prior tamoxifen or antiestrogen treatment exposure. HR+ patients can receive targeted endocrine therapy, such as aromatase inhibitors and selective estrogen-receptor response modulators. In the HR- subtype, HER-2 overexpressing patients usually receive anti-HER-2 targeted therapy such as Herceptin, followed by chemotherapy.

2.1.7 5-fluorouracil based chemotherapy

Since the advent of anti-folate based chemotherapy, anti-folate based agents such as methotrexate have remained widely used in the treatment of breast cancer [83]. Another chemotherapeutic agent, 5-fluorouracil (5-FU), is a prototype pyrimidine antagonist with several cytotoxic mechanisms that will be discussed in more detail in section 2.1.7.1.
Among several commonly used systemic combination adjuvant chemotherapy formulations, 5-FU is a common active ingredient. There are three main formulations containing 5-FU that are recommended by the NCCN and CCO for use in all three subtypes of breast cancer either as systemic chemotherapy, adjuvant chemotherapy, combination targeted therapy, and neo-adjuvant chemotherapy [4, 7]. The CMF formulation, containing cyclophosphamide, methotrexate and 5-FU, became the first standard therapy for early breast cancer patients, followed by FAC containing 5-FU, adriamycin, and cyclophosphamide and FEC made up of 5-FU, epirubicin, and cyclophosphamide [83].

In Canada, CCO recommends CMF formulations containing 600 mg/m² 5-FU as a standalone regimen for TNBC as well as in conjunction with trastuzumab for HER-2 positive patients. Importantly, at present, the standard adjuvant chemotherapy for breast cancers is **FEC-D (5-FU, epirubicin, cyclophosphamide-docetaxel) at 500 mg/m²**. Additionally, FEC containing 500 mg/m² 5-FU in combination with trastuzumab is a standard regimen widely used in HER-2 positive patients in Ontario [5].

<table>
<thead>
<tr>
<th>Breast Cancer Stage</th>
<th>Triple Negative ER-, PR-, HER2-</th>
<th>HER2 + ER-, PR-, HER2+</th>
<th>Luminal B ER+, PR+, HER2+</th>
<th>Luminal A ER+, PR+, HER2-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I, II, III chemotherapy</td>
<td>FEC-D CMF</td>
<td>FEC-D CMF</td>
<td>FEC-D CMF</td>
<td>FEC-D CMF</td>
</tr>
<tr>
<td>Stage IV chemotherapy</td>
<td>FEC-D CMF</td>
<td>FEC-D/ CMF + Trastuzumab</td>
<td>FEC-D/ CMF + Trastuzumab</td>
<td>FEC-D CMF</td>
</tr>
</tbody>
</table>

Table 2.1.7. Chemotherapy formulations used in the treatment of early and late stage breast cancer depending on subtype. The CMF formulation contains cyclophosphamide, methotrexate and 5-FU and the FEC-D formulation contains 5-FU, epirubicin, cyclophosphamide and docetaxel.
2.1.7.1 Mechanisms of 5-fluorouracil

5-FU is a prototype pyrimidine antagonist prodrug that structurally resembles uracil. As such, 5-FU must be converted intracellularly into cytotoxic nucleosides and nucleotides to produce its active cytotoxic metabolites. In humans, 80-90% of the administered dose is degraded to 5,6-dihydrofluorouracil (DHFU) by dihydropyrimidine dehydrogenase (DPD) so only a small amount can be converted into active cytotoxic metabolites [8, 92] (Figure 2.1.7.1).

![Figure 2.1.7.1 Metabolism of 5-fluorouracil.](image)

5-FU requires a series of catalytic conversions by intracellular enzymes to be converted into one of its three active metabolites: 5FUTP, 5FdUTP, and 5FdUMP. The primary cytotoxic mechanism of 5-FU is the formation of an inhibitory 5,10-methyleneTHF-TS-5FdUMP ternary complex, blocking de novo synthesis of pyrimidines. Secondary cytotoxic mechanisms include the incorporation of 5FUTP and 5FdUTP into RNA and DNA respectively, resulting in cell death through apoptosis-related mechanisms. Image adapted and reprinted by permission from the publisher (Nature Publishing Group) [92].
Active cytotoxic metabolites can be formed by three routes: conversion of 5FU to 5-fluoro-uridine-monophosphate (5FUMP) by orotate phosphoribosyl transferase; sequential conversion of 5FU to 5FUMP by uridine phosphorylase and uridine kinase; and sequential conversion of 5FU to 5-fluoro-deoxyuridine-monophosphate (5FdUMP) by thymidine phosphorylase and thymidine kinase [8, 92]. As illustrated in Figure 2.1.7.1, 5-FU had three potential cytotoxic mechanisms including incorporation of its metabolites 5-fluoro-deoxyuridine-triphosphate (5FdUTP) and 5-fluoro-uridine-triphosphate (5FUTP) as false precursors in DNA and RNA, respectively. Additionally, a third metabolite, 5FdUMP can form an inhibitory ternary complex with TS and 5,10-methyleneTHF, thereby inhibiting TS and suppressing DNA synthesis [8]. Once 5FdUTP and 5FUTP become misincorporated into DNA and RNA, respectively, it results in DNA instability and interference with RNA processing and function, respectively [8]. Consequently, these cancer cells become marked for p53-mediated apoptosis, resulting in cell death.

Since cancer cells are continually replicating, high stores of intracellular nucleotides are needed. When the inhibitory ternary complex is formed with TS, deoxythymidine monophosphate (dTMP) formation is blocked, thereby depleting intracellular stores critical for DNA synthesis in the rapidly dividing cancer cells. As such, this mechanism contributes to the high cytotoxicity of 5-FU observed in cancer.

Clinically, 5-FU is administered with LV, also called folinic acid or 5-formylTHF, which is a precursor of 5,10-methyleneTHF. LV potentiates the cytotoxic effects of 5-FU by directly contributing to the intracellular stores of 5,10-methyleneTHF, thereby enhancing the
formation and stabilization of the inhibitory 5,10-methyleneTHF-TS-SFdUMP ternary complex [8].

2.1.7.2 Mechanisms of acquired drug resistance to 5-FU

Generally, drug resistance to anti-folate based chemotherapeutics results from the following mechanisms: 1) impaired drug delivery and uptake due to the downregulation of reduced-folate carrier (RFC); 2) decreased folylpolyglutamate synthase (FPGS) and/or increased γ-glutamyl hydrolase (GGH) expression resulting in decreased polyglutamylation; 3) overexpression and mutations in TS and/or dihydrofolate reductase (DHFR) resulting in decreased affinity for cytotoxic metabolites; and 4) increased drug efflux due to a higher expression of multi-drug resistance pumps (MRPs) [93]. These mechanisms will be discussed in more detail in section 2.4.4.

Overexpression of TS has also been shown to reduce sensitivity to 5-FU. Omura and colleagues reported that upon repeated treatment with 5-FU, TS expression is increased, resulting in a significantly higher number of SFdUMP binding sites and a decreased sensitivity to 5-FU [94]. Similarly, another study by Kawakami and colleagues found that an activating polymorphism of the TS promoter caused a three-fold increase in TS mRNA levels and significantly lower chemosensitivity to 5-FU compared to the wildtype control [95]. Additionally, overexpression of DHFR can also reduce sensitivity to 5-FU, by increasing TS activity since the transcription of both TS and DHFR is co-regulated by several transcription factors [96].

Finally, ATP-binding cassette (ABC) transporters that mediate the efflux of various metabolites from the cell harnessing energy from ATP hydrolysis, play an important role in
multidrug resistance [97]. To date, ABC transporters are classified into seven families denoted by letters A-G depending on genetic homology and have 49 members [97]. These transporters are crucial in mediating the transfer of metabolic products through cellular membranes, including proteins and amino acids, lipids and sterols, ions and drugs such as 5-FU [97]. Specifically, MRP5 (ABCC5), MRP8 (ABCC11) and ABCB5 are implicated in 5-FU efflux [98]. However, ABCB5 is not expressed in breast tissue and so will not be examined in this thesis [98] (Table 2.1.7.2).

Table 2.1.7.2. List of selected ABC transporter proteins linked to multidrug resistance. MRP5 and MRP8, implicated in 5-FU resistance and expressed in breast tissue are indicated in red. Table adapted and reprinted by permission from the publisher (Oxford University Press) [97].
In addition to these three transporters, the study by Sadahiro and colleagues also demonstrated low MRP1 expression was predictive of high intracellular folate levels upon LV treatment [99]. Although MRP1 is associated with methotrexate resistance, MRP1 may modulate 5-FU efficacy through LV [98]. Other than exporting short chain polyglutamate forms of methotrexate, MRP1 may also regulate intracellular folate stores by exporting monoglutamate or short-chain polyglutamate forms of folate such as LV, resulting in a decrease in the efficacy of LV to potentiate the cytotoxicity of 5-FU [99].

Unlike MRP1, MRP5 is involved only in the efflux of one of the active cytotoxic metabolite of 5-FU, 5FdUMP, whereas MRP8 mediated the efflux of 5-FU, 5-fluoro-2'-deoxyuridine, and 5-fluoro-5'-deoxyuridine alike [98]. Several studies have shown increased resistance to 5-FU through increased efflux through MRP5 and MRP8 [100-103]. In an in vitro study, MRP8 transfected kidney cells exhibited overexpression of MRP8 that resulted in a 3 and 5-fold resistance to 5FdUMP and FUDR respectively, compared to wild type [101]. This observation was further supported by Oguri and colleagues who have demonstrated active 5FdUMP efflux by MRP8 in a 5-FU resistant cell line, resulting in a 25-fold increase in resistance to 5-FU [103]. Similarly, MRP5 transfected HEK cells were 9-fold more resistant to 5-FU compared to the wildtype [102].

To date, the mechanism of MRPs is poorly understood as MRPs have not only been implicated in resistance to 5-FU but also in increasing 5-FU efficacy. This dual effect appears to be modulated mostly by the total intracellular folate stores and the degree of polyglutamylation discussed in more detail in section 2.4.4.2 [98]. Since 5-FU is the cornerstone of chemotherapy formulations used in the treatment of breast cancer, it is
important to examine whether or not folate status can influence the aforementioned mechanisms of acquired drug resistance to 5-FU.

2.2 Folate

2.2.1 An overview of folate

Both folate, a naturally occurring water soluble B vitamin (vitamin B9), and its synthetic form, folic acid (FA), are critical for maintaining human health as they mediate one-carbon transfer reactions involved in nucleotide biosynthesis and biological methylation reactions [9]. In this role, folate is essential for DNA synthesis, integrity and repair as well as regulation of gene expression through DNA methylation [9]. As such, both folate deficiency and excess have been associated with a variety of adverse health outcomes. In humans, folate deficiency is associated with megaloblastic anemia, congenital disorders including neural tube defects (NTDs), cognitive impairment, elevated homocysteine levels which are associated with increased cardiovascular disease risk, and increased risk of certain cancers [10-12]. On the other hand, an emerging body of evidence suggests that folate excess, primarily from FA supplementation, may mask B12 deficiency, accelerate cognitive dysfunction, in conjunction with low vitamin B12 status, and promote the progression precancerous lesions [11].

2.2.1.1 Dietary sources and recommendations

Since humans cannot synthesize folate, humans rely on colonic bacterial production, diet and supplements for maintaining folate levels. The main source of folate is the diet; folate occurs naturally in a variety of foods, including but not limited to green leafy vegetables, asparagus, broccoli, Brussels sprouts, citrus fruits, dry cereals, whole grain, legumes, yeast,
liver and other organ meats as pteroylpolyglutamates in reduced forms [12]. The bioavailability of folate in these foods varies as naturally occurring folates are unstable and prone to oxidation [12]. An estimated 50-75% of folate content is lost thorough food preparation and storage [12]. On the other hand, FA, pteroylmonoglutamic acid, the synthetic, fully oxidized monoglutamyl form of folate that is used to fortify food and in supplements, is highly stable and readily bioavailable [104].

In order to account for differences in folate and FA bioavailability and stability, folate intake is usually measured in dietary folate equivalents (DFEs), where 1 DFE is equal to 1 µg of naturally occurring dietary folate, 0.6 µg of FA in fortified foods or in supplements ingested with food, or 0.5 µg of FA taken on an empty stomach [12]. Currently, the recommended dietary intake (RDA) of folate is set at 400 µg DFE/ day for adult men and women, 600 µg DFE/ day during pregnancy and 500 µg DFE/ day during lactation in North America with the tolerable upper limit (UL) for FA set at 1 mg of FA/ day [12]. No tolerable upper limit has been determined for the natural form of folate [12].

2.2.2 Chemical Structure

As illustrated in Figure 2.2.2, the chemical structure of folate consists of three moieties: an aromatic pteridine ring joined to para-aminobenzoic acid (PABA) through a methylene bridge, and glutamic acid joined to PABA through a γ-peptide bond [9]. The term folate refers to a variety of folate vitamers that share this characteristic chemical structure and nutritional properties [105]. The pteridine ring is usually reduced in naturally occurring folates, which renders them unstable and prone to oxidation. In contrast, the pteridine ring in FA is fully oxidized, making it more stable. Additionally, naturally occurring folates have a polyglutamate
chain, containing up to 9 glutamate residues, whereas FA is monoglutamylated enabling rapid absorption and higher bioavailability compared to naturally occurring folates [9].

Figure 2.2.2: Chemical structure of [A] folic acid and [B] folate. Folate consists of three moieties: an aromatic pteridine ring joined to para-aminobenzoic acid (PABA) through a methylene bridge, and glutamic acid joined to PABA via a γ-peptide bond. FA is fully oxidized and monoglutamylated, whereas naturally occurring folates are reduced and contain a polyglutamate chain consisting of up to 9 glutamate residues. One-carbon units can be linked to THF at the N5 and N10 positions: R = CH3 (N5), CHO (N5 & N10), CH=NH (N5), CH2 (N5 & N10) and CH= (N5 & N10). Adapted and reprinted by permission from the publisher (John Wiley and Sons Ltd) [10].

2.2.3 Folate absorption and metabolism

Naturally occurring folates have polyglutamate chains that must be hydrolysed to three or less glutamate residues in order to cross the cellular membrane [9]. The absorption of folate occurs mostly in the proximal small intestine, where an exopeptidase enzyme anchored in the intestinal apical brush membrane called glutamate carboxypeptidase II (GCPII) hydrolyzes the
polyglutamate chain [9]. Once hydrolyzed, there are three transporter, carrier and receptor expressed in the small intestine that can mediate folate absorption through the apical cellular membrane: proton-coupled folate transporter (PCFT), reduced folate carrier (RFC) and folate receptors (FR; where FR-α is the predominant isoform of FR in epithelial membranes) [9]. Of these three, PCFT is believed to be the primary transporter as it is able to carry out folate transport at a low pH found in the micro-environment of the small intestine and has equal affinity for folates regardless of the oxidation state of the pteridine ring [9]. In contrast, RFC has a higher affinity for reduced folates and is only optimal at physiological pH. Finally, FRs have the highest affinity for FA [9].

Once folate is taken up into the cell, it is polyglutamylated by the enzyme FPGS since polyglutamylated folates are better retained intracellularly and are better substrates for intracellular enzymes involved in one-carbon and folate metabolism [9]. Another enzyme, GGH, hydrolyzes polyglutamate chains, thereby mediating their efflux from the cell via RFC in the basolateral membrane of the cell [9]. Therefore, folates that enter the circulation are usually monoglutamylated and are transported unbound or bound to protein. Almost 50% of protein-bound folates are bound to albumin in the circulation, and a small proportion is bound to soluble folate receptors (mainly FR-γ) [9, 106]. Similar to intestinal absorption, folate is taken up into target tissue by the same transporter, carrier and receptor (PCFT, RFC, FR). Since target tissues express these receptors in different proportions, intracellular folate levels vary across different types of tissues [9].

Unlike naturally occurring folates, FA must be biotransformed to metabolically active 5′-methylTHF that is the predominant form of folate in the circulation. Biotransformation
occurs mostly in the liver (although it can also occur in the small intestine), and is carried out by DHFR, which reduces FA first to dihydrofolate (DHF) and then to tetrahydrofolate (THF) as shown in figure 2.2.3 [107]. Once THF is methylated, it yields 5’-methylTHF, which is critical for the regeneration of methionine and downstream methylation [107].

2.2.4 Biochemical functions of folate

As stated earlier, folate plays a critical role in one-carbon transfer reactions, which are fundamental in the regeneration of methionine, biological methylation reactions and nucleotide biosynthesis.

2.2.4.1 Regeneration of methionine

As illustrated in Figure 2.2.3, the enzyme methionine synthase (MS; 5-methyltetrahydrofolate-homocysteine methyltransferase) coupled with the co-factor cobalamin (vitamin B12) mediates the transfer of a methyl group from 5-methylTHF to homocysteine, thereby generating methionine and THF. Regenerating intracellular methionine stores is critical as methionine is the precursor of S-adenosylmethionine (SAM), the universal methyl group donor in most of biological methylation reactions including DNA methylation [9].

2.2.4.2 Biological methylation reactions

SAM is produced through the activation of methionine by adenosine triphosphate (ATP) and the action of methionine adenosyltransferase (MAT) [9]. With SAM as the methyl group donor, cytosine guanine dinucleotide (CpG) DNA methyltransferases (DNMTs) mediate DNA methylation, where DNMT1 is responsible for maintaining DNA methylation, while DNMT3a and DNMT3b mediate de novo DNA methylation of cytosines within the CpG sequences [108].
2.2.4.3 Nucleotide biosynthesis

As illustrated in Figure 2.2.3, folate also plays a critical role in pyrimidylate and purine biosynthesis needed for DNA synthesis [9]. THF and serine undergo a reversible conversion to 5,10-methyleneTHF and glycine through the action of serine hydroxymethyl transferase (SHMT). In a subsequent reaction catalyzed by the enzyme thymidylate synthase (TS), a methyl group from 5,10-methyleneTHF is transferred to deoxyuridine monophosphate (dUMP), generating deoxythymidine monophosphate (dTMP, thymidylate, a precursor of pyrimidylate biosynthesis) and DHF [9]. 5,10-methyleneTHF can also be channelled to purine biosynthesis [9].
Figure 2.2.3 Folate absorption, metabolism, and biochemical functions. Folates are hydrolyzed by glutamate carboxypeptidase II (GCPII), transported across the intestinal apical cellular membrane mostly by the proton-coupled folate transporter (PCFT) and to a lesser extent RFC and the folate receptor (FR). Intracellularly, folate is polyglutamylated by the enzyme folylpolyglutamate synthase (FPGS) and terminal glutamate residues are removed from polyglutamylated folates by the enzyme γ-glutamyl hydrolase (GGH). Folate plays a critical role in one-carbon transfer reactions, which are fundamental in regeneration of methionine, biological methylation reactions and nucleotide biosynthesis. Folate also plays a critical role in pyrimidylate and purine biosynthesis needed for DNA synthesis. THF and serine undergo a reversible conversion to 5,10-methyleneTHF and glycine through the action of serine hydroxymethyl transferase (SHMT). In a subsequent reaction catalyzed by the enzyme thymidylate synthase (TS), a methyl group from 5,10-methyleneTHF is transferred to deoxyuridine monophosphate(dUMP), generating deoxythymidine monophosphate (dTMP, thymidylate, a precursor of pyrimidylate biosynthesis) and dihydrofolate (DHF). If 5,10-methyleneTHF is not involved in pyrimidylate biosynthesis, 5,10-methyleneTHF coupled with formylated THF can also participate in the purine synthesis pathway. Adapted and reprinted by permission from the publisher (John Wiley and Sons Ltd) [10].
2.3 Folate and Health

2.3.1 Folate and health

Given the critical role of folate in the regeneration of methionine, biological methylation reactions and nucleotide biosynthesis, optimal folate levels are necessary to maintain human health. Folate deficiency can be caused by a variety of factors, such as inadequate dietary intake, increased folate demand (for instance in pregnancy), gastrointestinal disorders that affect folate absorption (e.g., celiac disease, inflammatory bowel disease), chronic smoking, alcohol consumption, and various medications such as anti-epileptic, anti-inflammatory and anti-folate drugs [12].

In humans, folate deficiency is associated with megaloblastic anemia, congenital disorders including NTDs, adverse pregnancy and birth outcomes, cognitive impairment, hyperhomocysteinemia which is purported to increase the risk of coronary heart disease and stroke, and increased cancer risk [10-12]. Except for the association between folate deficiency and the risk of NTDs and megaloblastic anemia, the exact nature and magnitude of relationship between folate deficiency and the aforementioned disorders have not been unequivocally established [13, 109, 110]. Furthermore, the effect of FA supplementation on the prevention and correction of the aforementioned disorders have been largely null except for the unequivocal protective effect of periconceptional FA supplementation on NTDs and the effective treatment of FA supplementation of megaloblastic anemia [12, 13]. Periconceptional FA supplementation appears to have a null effect on the risk of other congenital defects, although some studies have suggested a protective effect of periconceptional FA supplementation on congenital heart defects [111, 112]. Although not uniformly consistent,
data from randomized trials of FA supplementation generally suggests FA is protective against cognitive decline [113, 114]. On the other hand, several large randomized clinical trials as well as systematic reviews and meta-analyses have found that FA supplementation, either alone or in combination with other B vitamins, has no effect on secondary prevention of coronary heart disease in predisposed individuals despite significant reductions in homocysteine levels [115, 116].

Because folate is integrally involved in the generation of SAM (Figure 2.2.3), both folate deficiency and FA supplementation may lead to changes in intracellular SAM and consequent DNA methylation alterations. However, the effects of folate status on DNA methylation are highly complex; they are cell and tissue-specific and are dependent on the timing and duration of folate intervention. Furthermore, they depend on interactions with other methyl group donors (e.g., betaine, choline, vitamin B12, methionine) and other dietary factors as well as important genes involved in folate metabolism [117].

As DNA methylation is an epigenetic determinant for gene expression, stability, and aberrant patterns, dysregulation of DNA methylation is mechanistically related to the development of several chronic diseases including cancer [10]. DNA methylation of cytosines located within CpG sequences is the most well studied mechanism for both global and gene-specific DNA methylation [118]. Normal cells are characterized by genomic hypermethylation, where 80% of low density CpG sites found throughout the genome are methylated [64]. However, CpG rich areas that include the promoter region of approximately half of all transcribed genes in humans are unmethylated [118, 119]. This allows for the transcription of these genes in normal cells. In tumor cells, global and gene-specific patterns are reversed [118,
Tumor cells are characterized by global hypomethylation and gene-specific hypermethylation (methylation of promoter region CpG islands), resulting in the silencing of genes such as tumor suppressor and mismatch genes and other critical cancer-related genes.

DNA methylation changes resulting from altered folate status may predispose individuals to developing chronic diseases. Since FA supplementation results in increased SAM stores (Figure 2.2.3), supplemental FA can reverse pre-existing DNA hypomethylation, thereby protecting against neoplastic transformation [120]. On the other hand, high SAM stores from supplemental FA may also cause de novo CpG island methylation, resulting in gene-specific hypermethylation of genes such as tumor suppressor genes and increased cancer progression [10]. However, the effects of folate deficiency and folic acid supplementation on DNA methylation are highly complex as they are gene and site-specific and depend on species, cell type, target organ, and stage of transformation as well as on the timing, degree and duration of folate intervention [117]. A recent study has shown that high FA can inhibit MTHFR activity in vitro and reduce MTHFR protein levels in mice [121], which may lead to DNA hypomethylation. One possible mechanism for this observation is that high FA may cause DNA hypermethylation of the MTHFR promoter, resulting in lower MTHFR protein expression and activity.

More recently, concerns have been raised over the effect of periconceptional FA supplementation on DNA methylation programming of the developing fetus and consequent disease susceptibility later in life. In proof-of-concept animal experiments using the agouti and AxinFused mouse models, maternal supplementation of methyl group donors (betaine, choline, FA and vitamin B12) permanently altered respective phenotypes by increasing DNA
methylation in the promoter/regulator regions of the agouti and AxinFused genes [122, 123]. In agouti mice, the observed DNA methylation and phenotypic changes were associated with leaner offspring with a lower tendency towards cancer, diabetes and obesity [123]. Recent animal studies have also demonstrated that maternal FA supplementation alone could alter global and gene-specific DNA methylation in the offspring in a gestation-dependent and organ-specific manner [124-127]. Furthermore, maternal FA supplementation was associated with a decreased risk of colorectal cancer [124] and an increased risk of mammary tumors [126] in the offspring, in part through altered DNA methylation.

2.3.2 Folic acid fortification

A convincing body of evidence on periconceptional FA supplementation reducing risk of NTDs led to the implementation of mandatory FA fortification of white wheat flour in 53 countries, including the U.S. and Canada [13-15]. When mandatory fortification was introduced in Canada and the United States in 1998, Canadian white wheat flour and cornmeal was mandated to be fortified with 150 µg FA/100 g and enriched pasta with 200 µg/100 g [14]. In the U.S., FA fortification policy was more conservative, mandating fortification of white wheat flour, cornmeal and enriched pasta with 140 µg FA/100 g [15]. It is important to note that many European countries have not adopted mandatory FA fortification to avoid adverse effects, although voluntary fortification during pregnancy is encouraged [13]. Overall, mandatory FA fortification is considered a success, as in North America FA fortification resulted in a 15-50 % decrease in NTD incidence [13].
2.3.3 Folic acid supplementation

2.3.3.1 In the general population

In addition to consuming FA fortified foods, approximately 30-40 % of the North American population consume supplements that usually contain 400 µg of FA [16-19]. This may be due in part to policy advising all young women of child-bearing age to consume FA containing supplements. For instance, in Canada, Health Canada advises all pregnant women and women planning on conceiving, to take a daily supplement containing an additional 400 µg of FA [128]. Canadian women in this age group however, exceed their recommended 400 µg of FA intake since almost all prenatal multivitamin supplements contain 1 mg of FA (UL of FA) [129]. Further, studies have also found that supplement use is associated with the female sex, higher socioeconomic status, age and healthy mindset in the U.S. and Canada alike [17, 18]. Due to the potential but as yet unproven protective effects of FA against stroke, cognitive decline and the development of certain cancers, many people choose to voluntarily consume FA containing supplements. In addition to women of reproductive age, FA supplements ranging from 1-5 mg are prescribed to patients suffering from megaloblastic anemia, ulcerative colitis, liver disease, alcoholism and cognitive decline [11].

2.3.3.2 Among breast cancer patients and survivors

Multivitamin supplement use is prevalent in cancer patients and survivors; a recent meta-analysis found that 14-32 % of cancer patients initiate supplement use after diagnosis [22]. Of all cancer patients, breast cancer patients were found to have the highest prevalence of multivitamin use at 57-62% [22]. Similarly to the general population, the female sex, younger age, higher level of education and greater physical activity were found to be
consistently associated with supplement use in breast cancer patients [22]. A study that provided information on communication between patient and physician, 69% of supplement using breast cancer patients reported that their physician was informed of this [130]. Another study suggests supplement use is more common in breast cancer patients who were unsatisfied with their physician [131]. The prevalence of supplement use in breast cancer patients is of concern as the National Cancer Institute strongly advises patients to avoid vitamin use prior to and over the course of their treatment as it may adversely affect the efficacy of the treatment [132].

2.3.4 Blood levels of folate in the post-fortification era

At present, FA fortification of the food supply combined with widespread supplement use, usually containing 400 µg of FA, has resulted in significantly higher intake and blood levels of folate and FA in North America compared with the pre-fortification era [20, 21].

In the U.S., data from NHANES collected from 1988-2010 revealed a 1.5 times and 2.5 times increase in geometric mean concentrations of red blood cell folate (RBC; long term measurement of folate status) and serum (reflects short term dietary folate intake), respectively, in 1999-2010 (post-fortification era) compared to 1988-1994 (pre-fortification era) concentrations [20]. Data from the most recent Canadian Health Measures Survey (CHMS) from 2007-2009 indicated that less than 1% of the Canadian population was folate deficient (defined as RBC folate concentrations < 305 nmol/L [12]), whereas more than 40% of the population had RBC folate concentrations above an arbitrary high-concentration cut-off (defined as levels above the 97th percentile of RBC folate concentrations from NHANES 1999-2004 - 1360 nmol/L) [21].
As mentioned in section 2.2.3, since high levels of FA can saturate the enzyme DHFR, unaltered or unmetabolized FA can enter the circulation. Indeed, data from the Framingham Offspring Cohort found the prevalence of UMFA in the North American population was 74.7% post-fortification in comparison to 55.0% pre-fortification in supplement non-users and 80.7% post-fortification in comparison to 72.5% pre-fortification in supplement users [133]. A recent study using serum samples from NHANES 2007-2008, also found a significantly higher geometric mean concentration of UMFA in supplement users compared to non-users at 1.54 and 0.794 nmol/L, respectively [134]. Additionally, UMFA levels >0.3 nmol/L were detected in more than 95% of supplement users and non-users and UMFA concentrations of >1 nmol/L were detected 33.2% overall and 21.0% in fasting adults [134]. It is important to note that the increased prevalence of UMFA in the circulation may not necessarily be due to mandatory fortification of the food supply, since UMFA is also prevalent in countries such as Ireland where mandatory fortification has not been implemented [135].

However, a recent study conducted by Yeung and colleagues, using NHANES data collected from 2001-2004 in the U.S. found that 75% of participants in the highest serum folate concentration quintile were supplement users [136]. On average, supplements containing FA contributed 61% of the total daily FA intake, whereas enriched cereal-grain products only contributed 22% [136]. Therefore, this study demonstrated that the main driving force responsible for high serum folate concentrations is FA intake from supplements, not FA intake from fortification or enriched cereals. Also in the U.S. using 2001-2002 NHANES data, Bailey and colleagues found the group with detectable levels of UMFA had a significantly higher proportion of FA supplement users compared to the group without detectable UMFA [137].
Additionally, 40% of the group where UMFA was present was in the highest quartile of total FA intake [137]. Therefore, FA intake from supplements is not only the driving factor leading to high serum folate, but also the detectable UMFA levels observed in the U.S. population.

### 2.3.5 Potential adverse effects of high FA intake in the post-fortification era

As mentioned previously, high FA intake is associated with a variety of adverse outcomes such as masking of vitamin B12 deficiency in the elderly, accelerated cognitive impairment, and decreased natural killer cell cytotoxicity. High FA can also mediate potential epigenetic changes through DNA hypermethylation, promote the progression of (pre)cancerous lesions and tolerance or resistance to anti-folate drugs and 5-FU [11]. High FA intake may mask vitamin B12 deficiency because high FA levels will replenish nucleotide stores needed for nucleotide synthesis thereby preventing anemia [11]. Since anemia is the main clinical tool used to diagnose vitamin B12 deficiency, in cases of high FA intake, vitamin B12 deficiency is masked and a variety of adverse neurological outcomes such as spinal cord damage may progress due to the underlying deficiency in B12 [11].

Regarding cognitive function, results are inconsistent among randomized clinical trials of FA supplementation alone or in combination with other B vitamins; although predisposed individuals generally report improvements in mood and cognition with chronic supplementation [114]. Additionally, a recent study demonstrated that supplementation including 0.8 mg of FA/ day resulted in a reduction of atrophy in regions of the brain associated with cognitive decline and dementia [113]. Troen and colleagues also interrogated the association between folate intake and natural killer cell cytotoxicity in postmenopausal women in the U.S. post-fortification [138]. The authors reported no relation between total plasma
folate and natural killer cell cytotoxicity, but observed a highly significant inverse linear association between the amounts of UMFA in plasma and natural killer cell cytotoxicity [138]. Since UMFA levels are driven mainly by high FA intake from supplements, this study raises the possibility of high FA intake impairing natural killer cell cytotoxicity.

High FA intake may also mediate potential epigenetic changes through DNA hypermethylation and promote the progression of (pre)cancerous lesions. Generally, human observational studies have demonstrated a positive relationship between blood folate levels and genomic DNA methylation in lymphocytes and colon cells [117]. A number of recent studies have also reported associations between elevated folate levels and cancer risk. A large Swedish study reported the lowest concentrations of plasma folate were protective against colorectal cancer, whereas higher concentrations were associated with increased risk [139]. Similarly, another study found that folate concentrations in the top quartile were associated with increased prostate cancer risk in older men [140]. Therefore, high FA intake, particularly from supplements may promote the progression of (pre)cancerous lesions resulting in increased cancer risk.

Evidence from the literature also suggests that FA can compete with reduced folates for binding to folate-dependent enzymes as well as folate receptor/transporter/carryer [11]. High levels of FA in the circulation have been shown to downregulate of folate transporter and carrier involved in folate absorption through a negative feedback mechanism [11]. An in vitro study using a human intestinal and renal cell line cultured at high FA concentration of 100 μmol/L had reduced gene and protein expression of PCFT and RFC responsible for folate uptake, resulting in decreased FA uptake into the cell [141]. This finding is supported by an in
vivo study, where Wistar rats supplemented with 20 mg FA/kg diet (10 times the BDR or control) for 10 days had reduced protein expression of PCFT and RFC in the small intestine compared with those fed the BDR of FA, although no changes in gene expression were observed [142].

High FA intake can also saturate DHFR, allowing UMFA to enter the circulation [143, 144]. This is of concern as the pharmacodynamics properties of UMFA are unknown in humans [145]. Chronically high levels of UMFA in the circulation may disrupt one-carbon transfer reactions, which may lead to the development of chronic diseases such as cancer [11]. There is also evidence that high levels of FA can inhibit DHFR, thereby impeding the reduction of DHF to THF, a critical process needed to generate 5-methylTHF, leading to intracellular folate deficiency [105, 143]. Additionally, the increased intracellular pool of DHF has been shown to inhibit several important enzymes involved in the folate pathway such as TS and MTHFR [11].

Chronically high intakes of FA are associated with a variety of adverse outcomes, the mechanisms of which still need to be elucidated. Studies have shown that high FA can downregulate folate transporter and carrier, resulting in decreased intracellular folate uptake and the saturation of DHFR, causing high levels of UMFA to enter the circulation, the mechanistic implications of which are poorly understood. The inhibition of DHFR, TS and MTHFR by UMFA is of particular concern for the efficacy of drugs such as 5-FU whose mechanism depends in part on the action of these enzymes and adequate intracellular stores of 5,10-methyleneTHF. Additionally, high FA levels may increase cell proliferation by providing increased nucleotide stores needed for DNA synthesis and high FA may also lead to aberrant methylation patterns.
2.4 Folate and breast cancer

Given the critical role of folate in DNA synthesis and DNA methylation, disruption of which is mechanistically related to cancer development, a potential association between folate status and cancer risk has been under intense investigation. Indeed, a large body of experimental, epidemiologic and clinical studies suggests that folate deficiency in normal tissues predisposes them to neoplastic transformation while FA supplementation suppresses neoplastic initiation in normal tissues [11]. Epidemiologic studies have generally suggested an inverse association between folate status and the risk of several human malignancies including cancer of the colorectum, oropharynx, esophagus, stomach, pancreas, breast, cervix, ovary, and lungs and neuroblastoma and leukemia [10, 146]. However, the precise nature and magnitude of the inverse relationship have not been unequivocally established [11]. Furthermore, in vitro and animal studies have shown that folate deficiency and FA supplementation have an inhibitory and promoting effect on established neoplasms, respectively [147]. Folate deficiency induces ineffective DNA synthesis and in rapidly replicating cells, such as neoplastic cells, this will result in inhibition of tumor growth [11, 147]. As such, interruption of folate metabolism has been the basis for cancer chemotherapy using anti-folate agents and 5-FU [148]. In experimental models, folate deficiency has been shown to suppress progression and induce regression of pre-existing neoplasms [10]. In the 1940’s, FA supplementation was shown to accelerate the progression of leukemia in children with acute leukemia [149] and to cause rapid hematologic and clinical relapse in patients with chronic myeloid leukemia [149], the critical discovery that led to the development of antifolate-based chemotherapy [10].
The portfolio of evidence from *in vitro*, animal and clinical studies suggest, dual modulatory effects of folate on cancer development and progression depending on the stage of cell transformation at the time of folate intervention (table 2.4.2) [10, 30, 150]. Folate deficiency has an inhibitory, whereas FA supplementation has a promoting, effect on the progression of established (pre)neoplastic lesions [29, 151, 152]. In contrast, folate deficiency predisposes normal tissues to neoplastic initiation, and modest levels of FA supplementation suppress, whereas supraphysiologic supplemental doses enhance, neoplastic transformation in normal tissues [152-154]. The relationship between folate status and breast cancer risk has not been clearly defined as epidemiologic and experimental studies have yielded conflicting results [146, 155].

2.4.1 Current evidence

2.4.1.1 Epidemiologic Evidence

Epidemiologic evidence regarding the role of folate on breast cancer is inconclusive [156, 157]. Generally, case-control studies have reported an inverse relationship with dietary folate intake but not total folate intake, or a null association, with only one study reporting an increase in breast cancer risk [26, 156-158] (Table 2.4.1.1). Prospective studies are even more inconsistent; the majority have reported a null association, three have reported an increased risk and six have reported an inverse association [155]. In a recent review by Kotsopoulos and Kim, a thorough summary is provided for prospective and case-control studies to date investigating folate intake and breast cancer risk [155] (Table 2.4.1.2). It is important to note that the protective effect of folate is more consistent among women who regularly consume alcohol, a folate antagonist [146]. Based on the published epidemiologic studies, two meta-
analyses were conducted by Lewis and Larsson: prospective studies found no significant relationship, whereas case-control studies found a significant, albeit small, inverse association, between folate intake and breast cancer risk [156, 157]. In a more recent meta-analysis performed by Larsson and colleagues involving 9 prospective and 14 case-control studies conducted in North America, Europe and Australia [157], neither dietary nor total folate intake was associated with risk of breast cancer in prospective studies but a significant inverse association was observed between dietary folate intake and breast cancer risk in case-control studies (OR = 0.80, 95% CI = 0.72 to 0.89) (Figure 2.4.1.1) [157].

The inconsistency in the epidemiological evidence may be explained in part by the multifactorial etiology of breast cancer and how breast cancer risk may be modified by the interaction of other dietary factors such as B6 or B12 [159, 160], molecular subtype of breast cancer (HR status) [161], menopausal status [161] and mutations in genes involved in the folate pathway [146]. In addition, these discrepancies may also arise due a variety of limitations in experimental design, such as our inability to correct for potential confounding factors, small sample size, uncertain biological induction time, genetic variability and problems inherent in dietary assessments.

Even though the evidence on the association of folate and breast cancer risk is inconclusive, recent epidemiologic studies have reported an increased risk of breast cancer with high folate levels [23-28]. Among postmenopausal women participating in the screening arm of the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial, a small but significant increase in breast cancer risk was reported among women in the highest compared with the lowest quintile of supplemental FA (≥400 vs. <400 μg/day) and total folate intake.
(>853 vs. ≤335.5 μg/day) (HR=1.19; 95%CI 1.04-1.41; and HR=1.32; 95%CI 1.04-1.68;
respectively) [23]. Additionally, in the prospective Women’s Health Study, Lin et al. reported a
trend for increasing risk with increasing total folate intake (Relative risk (RR)=1.24; 95%CI 0.88-
1.76) [25]. In the Nurses’ Health Study involving 88,774 participants, Zhang and colleagues
found that dietary folate intake was associated with a small but significant increased risk of
developing ER+ tumors relative to ER- [27]. The RRs comparing the highest (>332 μg/day) vs.
the lowest (<206 μg/day) quintiles were 1.15 (95%CI 1.01-1.30) and 0.97 (95%CI 0.79-1.18) for
ER+ and ER– tumours, respectively [27].

The temporal association between FA fortification (high folate status) and an increased
breast cancer risk was suggested by the finding that the increased breast cancer risk with high
plasma folate levels was strongest in the analysis limiting to follow-up after mandatory FA
fortification (≥1998 vs. <1998) [25]. Additionally, in the Cancer Prevention Study II Nutrition
Cohort, Stevens et al. demonstrated a positive association between the highest quintile of
dietary folate intake (≥312.2 μg/day) and the risk of postmenopausal breast cancer compared
to the lowest quintile (<166.9 μg/day) (RR=1.12; 95%CI 1.01-1.24) [24].
Table 2.4.1.1. Summary of prospective studies interrogating the relationship between folate intake and breast cancer risk.

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Participants, age at enrollment, study period, country</th>
<th># of cases</th>
<th>Folate intake/source</th>
<th>Specific target group</th>
<th>RR, HR (95 % CI)</th>
<th>p-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhang et al.</td>
<td>88,818 34–59 years NHS 1980–1996 (United States)</td>
<td>3,483</td>
<td>Total folate (µg/d) 150–299 versus 600≤</td>
<td>All</td>
<td>0.93 (0.83–1.03)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Rohan et al.</td>
<td>5,382 NHSS 1980–1993 (Canada)</td>
<td>1,336  ≤225 versus 354≤</td>
<td>All</td>
<td>Premenopausal</td>
<td>0.99 (0.79–1.23)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Sellers et al.</td>
<td>34,393 55–69 years IWH 1986–1999 (United States)</td>
<td>1,875  ≤294 versus ≤172</td>
<td>Postmenopausal: ER+</td>
<td>1.06 (0.82–1.36)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Postmenopausal: ER−</td>
<td>1.21 (0.98–1.54)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total folate (µg/d) 351≤ versus ≤186</td>
<td>Postmenopausal: ER+</td>
<td>1.04 (0.82–1.31)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Postmenopausal: ER−</td>
<td>1.14 (0.66–1.95)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Cho et al.</td>
<td>90,655 26–46 years NHS II 1991–1999 (United States)</td>
<td>714  ≤210 versus 420≤</td>
<td>Dietary folate (µg/d)</td>
<td>Premenopausal</td>
<td>1.03 (0.81–1.32)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total (µg/d) ≤228 versus 826≤</td>
<td></td>
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</tr>
<tr>
<td>Feigelson et al.</td>
<td>66,561 40–87 years CPS-II 1992–1998 (United States)</td>
<td>1,303  ≤179 versus 294≤</td>
<td>Dietary folate (µg/d)</td>
<td>Postmenopausal</td>
<td>1.07 (0.91–1.27)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total (µg/d) ≤210 versus 604≤</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baglietto et al.</td>
<td>17,477 40–69 years MCC 1990–2003 (Australia)</td>
<td>537  Per 100 µg/d increment in folate intake</td>
<td>All</td>
<td>1.01 (0.93–1.10)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Zhang et al.</td>
<td>88,744 34–59 years NHS 1980–2000 (United States)</td>
<td>4,422  ≤206 versus 337≤</td>
<td>Dietary folate (µg/d)</td>
<td>ER+</td>
<td>1.15 (1.01–1.30)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Total (µg/d) ≤228 versus 534≤</td>
<td>ER−</td>
<td>0.97 (0.79–1.21)</td>
<td>n.s.</td>
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<td></td>
<td></td>
<td></td>
<td>Postmenopausal: ER+</td>
<td>1.00 (0.89–1.14)</td>
<td>n.s.</td>
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<td></td>
<td>Postmenopausal: ER−</td>
<td>0.81 (0.66–1.09)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Lajous et al.</td>
<td>62,739 43–77 years E3 N 1993–2002 (France)</td>
<td>1,812  ≤296 versus 522≤</td>
<td>Postmenopausal</td>
<td>0.78 (0.67–0.90)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Stozenberg-Solomon et al.</td>
<td>25–69 years PLCO cohort 1993–2001 (United States)</td>
<td>691  ≤234 versus 337≤</td>
<td>Postmenopausal</td>
<td>0.98 (0.78–1.24)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total folate (µg/d) ≤335.5 versus 853≤</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted and reprinted by permission from publisher (Springer) [155].
Table 2.4.1.1. Summary of prospective studies interrogating the relationship between folate intake and breast cancer risk continued Adapted and reprinted by permission from publisher (Springer) [155].

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Participants, age at enrollment, study period, country</th>
<th># of cases</th>
<th>Folate intake/source</th>
<th>Specific target group</th>
<th>RR, HR (95 % CI)</th>
<th>p-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cui et al.</td>
<td>68,132, 50–79 years WHI 1993–2005 (United States)</td>
<td>1,792*</td>
<td>Dietary folate (µg/d)</td>
<td>Postmenopausal</td>
<td>1.09 (0.95–1.25)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;320 versus &gt;350</td>
<td>Postmenopausal</td>
<td>1.04 (0.91–1.20)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total folate (µg/d)</td>
<td>Postmenopausal</td>
<td>1.04 (0.91–1.20)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;351 versus &gt;748</td>
<td>Postmenopausal</td>
<td>1.04 (0.91–1.20)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Ericson et al.</td>
<td>11,699, ≥50 years MDC cohort 1991–2003 (Sweden)</td>
<td>392</td>
<td>Dietary folate (µg/d)</td>
<td>Postmenopausal</td>
<td>0.56 (0.35–0.90)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;153 versus 302</td>
<td>Postmenopausal</td>
<td>0.59 (0.36–0.97)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total DFE (µg/d)</td>
<td>Postmenopausal</td>
<td>0.59 (0.36–0.97)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;160 versus 582</td>
<td>Postmenopausal</td>
<td>0.59 (0.36–0.97)</td>
<td>0.01</td>
</tr>
<tr>
<td>Duffy et al.</td>
<td>88,530, 50–79 years WHI-OS 1993–2004 (United States)</td>
<td>1,783</td>
<td>Total folate (µg/d)</td>
<td>Postmenopausal</td>
<td>0.97 (0.84–1.12)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤228 versus ≥643</td>
<td>Postmenopausal</td>
<td>0.97 (0.84–1.12)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Kabat et al.</td>
<td>89,835, 40–59 years NBSS 1980–2001 (Canada)</td>
<td>2,491</td>
<td>Dietary folate (µg/d)</td>
<td>All</td>
<td>1.02 (0.90–1.17)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;237 versus ≥374</td>
<td>All</td>
<td>1.02 (0.90–1.17)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Larsson et al.</td>
<td>61,433, 42–73 years SMC Cohort 1987–2007 (Sweden)</td>
<td>2,952</td>
<td>Dietary folate (µg/d)</td>
<td>All</td>
<td>1.01 (0.90–1.13)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤277 versus ≥277</td>
<td>All</td>
<td>1.01 (0.90–1.13)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤200 versus ≥277</td>
<td>All</td>
<td>1.01 (0.90–1.13)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lin et al.</td>
<td>1,696, ≥45 years WHS 1993–2004 (United States)</td>
<td>848</td>
<td>Dietary folate (µg/d)</td>
<td>All</td>
<td>0.44 (0.52–1.37)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤248 versus ≥380</td>
<td>All</td>
<td>0.44 (0.52–1.37)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤263.9 versus ≥582</td>
<td>All</td>
<td>0.44 (0.52–1.37)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total (µg/d)</td>
<td>All</td>
<td>0.44 (0.52–1.37)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Xu et al.</td>
<td>1,508†, 25–98 years 1996–2002 LBCS (United States)</td>
<td>1,508</td>
<td>Dietary folate (µg/d)</td>
<td>All</td>
<td>0.81 (0.47–1.39)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤194 versus ≥301</td>
<td>All</td>
<td>0.81 (0.47–1.39)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total folate (µg/d)</td>
<td>All</td>
<td>0.81 (0.47–1.39)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤291 versus ≥869</td>
<td>All</td>
<td>0.81 (0.47–1.39)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Marat et al.</td>
<td>35,023, 50–76 years VITAL study, 2003–2006 (United States)</td>
<td>743</td>
<td>Dietary folate (µg/d)</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>210 versus 541</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total DFE (µg/d)</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>325 versus 1,022</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 years average total DFE (µg/d)</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>345 versus 1,272</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Posmenopausal</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ER+</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ER−</td>
<td>Postmenopausal</td>
<td>0.91 (0.68–1.22)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.38 (0.38–0.80)</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stevens et al.</td>
<td>70,656, 50–74 years CPS-II 1992–2005 (United States)</td>
<td>3,898</td>
<td>Dietary folate (µg/d)</td>
<td>Postmenopausal</td>
<td>1.12 (1.01–1.24)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤167 versus ≥312</td>
<td>Postmenopausal</td>
<td>1.12 (1.01–1.24)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤192 versus ≥919</td>
<td>Postmenopausal</td>
<td>1.12 (1.01–1.24)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Table 2.4.1.2. Summary of prospective and nested case–control studies investigating the relationship between blood folate levels and breast cancer risk.

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Participants, age at enrollment, study period, country</th>
<th># of cases</th>
<th>Blood folate levels</th>
<th>Specific target group</th>
<th>RR, HR (95 % CI)</th>
<th>p-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wu et al.</td>
<td>390 (1:1 matching) 18–90 years 1974–1994 (2 cohorts of women donated blood) (United States)</td>
<td>195</td>
<td>Serum folate (ng/mL)</td>
<td>Lowest versus highest quintile</td>
<td>All (1974 cohort) 1.08 (0.50–2.37) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18–90 years 1974–1994 (2 cohorts of women donated blood) (United States)</td>
<td></td>
<td></td>
<td></td>
<td>All (1989 cohort) 0.79 (0.33–1.90) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18–90 years 1974–1994 (2 cohorts of women donated blood) (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Pre-post (1974 cohort) 1.57 (0.49–4.96) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18–90 years 1974–1994 (2 cohorts of women donated blood) (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Pre-pre (1989 cohort) 0.89 (0.10–7.70) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18–90 years 1974–1994 (2 cohorts of women donated blood) (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Post-post (1974 cohort) 0.66 (0.17–2.60) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18–90 years 1974–1994 (2 cohorts of women donated blood) (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Post–post (1989 cohort) 0.67 (0.26–1.72) n.s.</td>
<td></td>
</tr>
<tr>
<td>Zhang et al.</td>
<td>1,424 (1:1 matching) 34–59 years NHS 1989–1996 (United States)</td>
<td>712</td>
<td>Plasma folate (ng/mL)</td>
<td>Highest versus lowest quintile</td>
<td>All 0.73 (0.50–1.07) 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,424 (1:1 matching) 34–59 years NHS 1989–1996 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Premenopausal 0.65 (0.26–1.65) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,424 (1:1 matching) 34–59 years NHS 1989–1996 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Postmenopausal 0.75 (0.49–1.15) n.s.</td>
<td></td>
</tr>
<tr>
<td>Rossi et al.</td>
<td>1,024 (1:1 matching) 40–90 years 1969–2001 (Australia)</td>
<td>15</td>
<td>Increments of plasma or RBC folate: 2 µg/L decrease in plasma folate 100 µg/L decrease in RBC folate</td>
<td>Breast cancer mortality</td>
<td>1.30 (0.77–2.17) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,024 (1:1 matching) 40–90 years 1969–2001 (Australia)</td>
<td></td>
<td></td>
<td></td>
<td>Breast cancer morbidity 0.98 (0.65–1.47) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,024 (1:1 matching) 40–90 years 1969–2001 (Australia)</td>
<td></td>
<td></td>
<td></td>
<td>0.96 (1.22–3.12) &lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Lin et al.</td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td>848</td>
<td>Plasma folate (ng/mL)</td>
<td>≤5.1 versus &gt;15.8</td>
<td>All 1.42 (1.00–2.02) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Premenopausal 1.99 (1.01–3.93) 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Postmenopausal 1.24 (0.81–1.90) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Pre-fortification (&lt;1998) 1.30 (0.73–2.32) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>Post-fortification (≥1998) 1.54 (1.02–2.31) 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>ER+ 1.74 (1.20–2.53) 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>ER− 0.82 (0.33–2.04) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>PR+ 1.71 (1.15–2.55) 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,696 (1:1 matching) ≥45 years WHS 1993–2004 (United States)</td>
<td></td>
<td></td>
<td></td>
<td>PR− 0.97 (0.48–1.94) n.s.</td>
<td></td>
</tr>
<tr>
<td>Ericson et al.</td>
<td>612 (1:2 matching) 50–82 years Malmo diet and cancer study 1991–2004 (Sweden)</td>
<td>204</td>
<td>Plasma folate (nmol/L)</td>
<td>≤6.0 versus ≥17</td>
<td>All 1.38 (0.87–2.10) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>612 (1:2 matching) 50–82 years Malmo diet and cancer study 1991–2004 (Sweden)</td>
<td></td>
<td></td>
<td></td>
<td>ER+ 1.33 (0.49–3.59) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>612 (1:2 matching) 50–82 years Malmo diet and cancer study 1991–2004 (Sweden)</td>
<td></td>
<td></td>
<td></td>
<td>ER− 0.82 (0.46–1.44) n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>612 (1:2 matching) 50–82 years Malmo diet and cancer study 1991–2004 (Sweden)</td>
<td></td>
<td></td>
<td></td>
<td>ER+ 2.67 (1.44–4.92) 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Adapted and reprinted by permission from publisher (Springer) [155].
Figure 2.4.1.1 Relative risks (RRs; in prospective studies) or odds ratios (ORs; in case–control studies) of breast cancer comparing the highest with the lowest dietary folate intake categories. Squares indicate study-specific risk estimates (size of the square reflects the study-specific statistical weight, i.e., the inverse of the variance); horizontal lines indicate 95% confidence intervals (CIs); diamond indicates summary estimate with its corresponding 95% confidence interval. Adapted and reprinted by permission from publisher (Oxford University Press) [157].

2.4.1.2 Animal Studies

In 1945, Leuchtenberger and colleagues provided the earliest evidence that folate can modulate breast cancer using an in vivo mouse model, where the intravenous injection of FA resulted in complete regression of spontaneous mammary tumors in mice [162, 163]. Almost 50 years later, three animal studies, two led by Kotsopoulos et al have shown that folate deficiency suppresses N-Methyl-N-nitrosourea (MNU) induced mammary tumor development, whereas FA supplementation had no effect [164-166]. Similarly, Baggott and colleagues reported that cancer multiplicity (defined as the number of mammary cancers per number of
tumor bearing animal) after 180 days of MNU injection was 1.32, 1.90, 2.14, and 2.73 in the FA 0 mg, FA 2 mg, FA 40 mg and LV 20 mg per kg diet groups, respectively [164]. The FA deficient group (0 mg FA), had significantly lower cancer multiplicity compared to the other three groups [164]. Therefore, similar to the study conducted by Kotsopoulos et al, this data is suggestive of FA deficiency suppressing MNU-induced mammary tumor initiation and progression in rats. However, unlike the Kotsopoulos study, this data suggests FA supplementation enhances MNU-induced mammary tumor initiation and progression. This observation is supported by a recent study by Manshadi and colleagues, who reported that FA supplementation between 2.5x and 5x the BDR in rats significantly increased the progression of existing DMBA-induced mammary neoplasms [29].

Recent animal studies have also demonstrated that maternal FA supplementation alone could alter global and gene-specific DNA methylation in the offspring in a gestation-dependent and organ-specific manner [124-127]. Interestingly, a recent study by Sie et al demonstrated that FA supplementation to the mother in utero and during lactation reduced the number of terminal end buds, a reliable biomarker of mammary tumor risk at adulthood in rodent models [167], in the offspring, suggesting a lower mammary tumor risk in the offspring with higher prenatal and postnatal folate exposure [168]. In contrast, the same group demonstrated that both maternal and postweaning FA supplementation that parallels the average post-fortification folate intake in North America in rats increased the risk of DMBA-induced mammary tumors in the offspring [126]. Ly et al also subsequently demonstrated that pups from dams supplemented with 2.5x the control (5 mg/kg FA diet) in late gestation or throughout pregnancy had 15–25% lower ER-α expression in the liver compared with other
groups of pups [125]. This finding was supported by an earlier finding by our group, whereby at 14 weeks of age, postweaning, but not maternal, FA supplementation significantly increased CpG promoter DNA methylation of the ER-α gene (p < 0.05) [127], which would suppress ER-α gene expression. Therefore, although evidence from animal studies is not conclusive, these studies do suggest that folate status can modulate mammary tumor development in part through estrogen signaling.

2.4.1.3 Randomized Clinical Trials

No randomized clinical trials (RCTs) have directly assessed the effect of FA supplementation on breast cancer risk. However, several RCTs that were designed to investigate the effect of FA supplementation on cardiovascular disease as the primary outcome have assessed the effect of FA supplementation on cancer risk as the secondary outcome, yielding conflicting results [115, 169]. Meta-analyses of these RCTs in turn, have reported either a cancer promoting or a null effect on cancer [115, 169-171]. A meta-analysis of 10 RCTs (n=38,233) by Wien et al in 2012 demonstrated that FA supplementation increased the risk of all cancer by 7% with a relative risk (RR) of 1.07 (95%CI 1.00-1.14) but a null effect was observed on breast cancer risk with a RR of 0.86 (95%CI 0.65-1.14) [170].

Recently, a meta-analysis of 15 RCTs to date was conducted, including 13 reporting on total cancer incidence, 6 reporting on total cancer mortality and only 7 reporting on site-specific cancer [171]. Qin et al reported that FA supplementation had no effect on total cancer incidence with a RR of 1.05 (95% CI 0.99-1.11) and no effect on breast cancer incidence with a RR of 0.82 (95%CI 0.63-1.07) [171].
Collectively, epidemiological and animal studies and RCTs provide inconclusive evidence concerning the role of folate in breast cancer development. However, given recent data from epidemiologic and animal models, suggesting FA supplementation enhances mammary tumor initiation and progression, further studies are warranted to elucidate the relationship between folate and breast cancer risk and the underlying mechanisms. Better understanding of underlying mechanisms would allow for better preventable strategies in women at risk and improved efficacy of anti-folate based chemotherapeutics and 5-FU used in the treatment of breast cancer.

2.4.2 Folate and its dual modulatory role in breast carcinogenesis

Some studies from the literature suggest an inverse relationship between folate status and cancer risk [10], whilst there is also evidence that FA supplementation may promote cancer progression and increase overall cancer risk [146]. This has been best illustrated in colorectal cancer where epidemiologic, animal studies and clinical trials have demonstrated the dual modulatory role of folate in colorectal carcinogenesis [10, 154]. In breast cancer, however, the relationship between folate and breast cancer risk is inconclusive. Recent epidemiologic studies have reported increased risk of breast cancer with high folate levels [23-28]. Recent animal studies suggest that FA supplementation that parallels the average post-fortification folate intake in North America in rats increased the risk of chemically induced mammary tumors in the offspring [126], and FA supplementation between 2.5x and 5x the BDR in rats significantly increased the progression on existing mammary neoplasms [29]. In contrast, recent meta-analyses of RCTs have reported that FA supplementation had no effect on breast cancer risk [170, 171].
The dual effects of folate on carcinogenesis depend on the stage of cell transformation at the time of folate intervention as well as the dose of folate [10]. In established neoplastic lesions, folate deficiency inhibits further progression and FA supplementation enhances the progression of these lesions [10]. Conversely, in normal tissue, folate deficiency increases the risk of neoplastic transformation, while modest supplemental levels of FA suppress, but supraphysiologic levels enhance, the development of neoplastic lesions [10].

2.4.2.1 Effects of folate deficiency and FA supplementation in (pre)neoplastic breast tissue

As stated earlier, in established (pre)neoplastic lesions, folate deficiency inhibits further progression of these lesions by limiting the availability of nucleotide substrates for DNA synthesis, resulting in the inhibition of tumor growth. Folate deficiency also decreases stores of SAM, which may reverse the hypermethylation of CpG islands of promoters of tumor suppressor genes [150].

In contrast, FA supplementation can promote the growth of (pre)neoplastic lesions in breast tissue by 1) increasing nucleotide substrates needed for DNA synthesis leading to tumor growth and 2) promoting de novo methylation of CpG islands of tumor suppressor genes and consequent gene silencing [150].
Table 2.4.2. Dual modulatory role of folate in carcinogenesis.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Folate Deficiency</strong></td>
<td><strong>Tumor promoting</strong> mechanism:</td>
<td><strong>Tumor inhibitory</strong> mechanism:</td>
</tr>
<tr>
<td></td>
<td>1. DNA strand breaks</td>
<td>1. Ineffective DNA synthesis</td>
</tr>
<tr>
<td></td>
<td>2. Impaired DNA repair</td>
<td>2. Reversal of promoter CpG island hypermethylation</td>
</tr>
<tr>
<td></td>
<td>3. Increased mutagenesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. Genomic DNA hypomethylation</td>
<td></td>
</tr>
<tr>
<td><strong>Folic Acid</strong></td>
<td><strong>Tumor inhibitory</strong> mechanism:</td>
<td><strong>Tumor promoting</strong> mechanism:</td>
</tr>
<tr>
<td><strong>Supplementation</strong></td>
<td>1. DNA stability and integrity</td>
<td>1. Provision of nucleotides for proliferation</td>
</tr>
<tr>
<td></td>
<td>2. Optimal DNA repair</td>
<td>2. <em>De novo</em> methylation of promoter CpG islands</td>
</tr>
<tr>
<td></td>
<td>3. Decreased mutagenesis</td>
<td>3. Hypermutability of CH$_3$-CpG</td>
</tr>
<tr>
<td></td>
<td>4. Prevention of aberrant DNA methylation</td>
<td></td>
</tr>
</tbody>
</table>

Table adapted, modified and reprinted by permission from the publisher (John Wiley and Sons) [10].

### 2.4.2.2 Effects of folate deficiency and FA supplementation in normal breast tissue

The effect of folate on normal breast tissue is the opposite of the effect on (pre)neoplastic breast tissue. FA supplementation on normal breast tissue is protective against the development of neoplastic lesions by 1) supplying an optimal amount of nucleotide substrates for DNA synthesis, resulting in DNA stability and integrity, decreased mutagenesis and optimal DNA repair and 2) by providing an adequate amount of methyl group to generate SMA, resulting in the prevention global DNA hypomethylation [150]. In contrast, folate deficiency promotes (pre)neoplastic transformation through increased DNA strand breaks due to uracil misincorporation [172, 173], impaired DNA repair [174], increased mutagenesis and genomic hypomethylation, resulting in the activation of genes that favor neoplastic transformation [174].

57
2.4.3 Potential modulatory effects of folate on chemosensitivity to 5-fluorouracil chemotherapy

At present, the optimal folate concentration which can both minimize undesired toxicity and enhance the efficacy of 5-FU chemotherapy is unknown. There are only a few studies that have directly examined the effect of folate status on the sensitivity of cancer cells to 5-FU with conflicting results (Tables 2.4.3.1 and 2).

Several studies have reported that folate deficiency increases chemosensitivity to 5-FU [32-34]. In an in vitro study, Backus and colleagues reported that human colon cancer cell lines (C26-A, C26-10, C26-G and WiDr) and squamous cell carcinoma of the head and neck (HNSCC) cell lines (11B, 14C and 22B) cultured in folate deficient medium containing 0.5, 1.0, and 2.5 nM of FA had a 2-5 fold higher chemosensitivity to 5-FU alone or in combination with LV compared to cells cultured in 8 µM FA (p<0.05) [32]. This difference in sensitivity was attributed to increased RFC activity in the folate deficient cells and up to 7-fold higher expression of TS in cells grown in folate rich medium compared to folate deficient cells [32]. Similarly, Tucker et al reported that treatment with 5-FU in Apc (Min/+)) mice that are genetically predisposed to intestinal neoplasia fed a no FA (0 ppm) diet not only induced regression of pre-existing tumors, but also inhibited post-therapeutic rebound in tumor burden tumor [33]. On the other hand, in mice consuming a diet supplemented with FA, treatment with 5-FU only resulted in the suppression of the expected increase in tumor burden and no regression of pre-existing tumors. Additionally, the mice consuming the highest level of FA in the diet (6 ppm) treated with 5-FU exhibited a tumor burden nearly identical to that in untreated age-matched controls six weeks after termination of therapy [33]. In another in vivo
model, C3H mouse mammary adenocarcinomas growth was suppressed approximately 80% when FU was administered to folate depleted mice 1 hr after LV administration, relative to approximately 50% suppression in control mice [34]. Collectively, these findings suggest FA deficiency creates an environment that enhances 5-FU cytotoxicity (Table 2.4.3.1).

Conversely, there is conflicting evidence regarding the effect of FA supplementation on the sensitivity of cancer cells to 5-FU (Table 2.4.3.2). Two in vivo studies suggest that FA supplementation can increase the sensitivity of breast cancer cells to 5-FU. Branda et al conducted an in vivo study using Weanling female Fischer 344 rats, where rats were placed on either a low, control or high FA diets and received 5-FU, cyclophosphamide and doxorubicin treatments [35]. Consistently higher tumor inhibition was reported in animals receiving the high FA diet compared to the low FA diet, suggesting that mammary tumors were more responsive to 5-FU in a folate rich environment [35]. Another in vivo study conducted by Raghunathan et al found that 5-FU alone suppressed C3H mammary adenocarcinoma growth by 25%, whereas FA in combination with 5-FU increased growth suppression to over 70% [36].

It is important to note that in this study mice were kept on a folate deficient diet so the results only indicate the acute, short-term repletion effects of FA supplementation. Another recent in vivo study examined the effect of FA supplementation on 5-FU chemosensitivity of human colon cancer cells. In a mouse human HCT116 colon cancer xenograft model, mice were fed control, 4× or 12.5× supplemental levels of FA in their diet [37]. Within each diet group, mice were further randomized to receive 5-FU+leucovorin or saline. In the control group 5-FU significantly inhibited the growth of the xenografts. However, at the 4× supplemental level, 5-FU-treated xenografts grew faster than untreated while at the 12.5× supplemental level, 5-FU
exhibited no cytotoxic effect [37]. These observations suggest that FA supplementation decreases the efficacy of 5-FU treatment.

Studies have also indirectly examined the modulatory role of folate status on 5-FU sensitivity by manipulating critical enzyme levels that influence intracellular folate concentrations, distributions and metabolism, such as MTHFR, FPGS and GGH [175-177]. Sohn and colleagues, using a xenograft model in mice, demonstrated a 78 % inhibition of xenograft growth of human colon cancer HCT116 cells having the MTHFR C677T polymorphism compared to the 36 % inhibition in the cells with the wild-type MTHFR genotype [175]. The C677T polymorphism causes inactivation of MTHFR, leading to higher intracellular stores of 5,10-methyleneTHF, which increases the formation and stabilization of the TS-5,10 methyleneTHF-5FdUMP ternary inhibitory complex, thereby increasing 5-FU chemosensitivity.

In an in vitro model of FPGS overexpression and inhibition in MDA-MB-435 breast cancer cells, Cho et al reported FPGS overexpression was associated with decreased chemosensitivity of breast cancer cells to 5FU + LV and 5FU at a high FA concentration [176]. In contrast, at low FA concentration in the medium, FPGS overexpression was associated with enhanced chemosensitivity to 5FU +LV, but not to 5FU alone [176]. FPGS overexpression would result in increased intracellular folate concentrations due to increased polyglutamylation, which enhances intracellular folate retention. Findings in this study highlighted the complex modulatory mechanism involving exogenous folate stores, enzyme expression and sensitivity to 5-FU. In an in vitro model, Kim et al showed GGH overexpression significantly decreased chemosensitivity of MDA-MB-435 cells to 5FU at all concentrations of folate, whereas in the HCT116 cell line, decreased chemosensitivity was only observed at the highest folate
concentration [177]. The overexpression of GGH results in increased hydrolysis of the polyglutamate chains and increased efflux of folate from the cell; essentially GGH overexpression depletes the cell of intracellular folate stores similar to the effect of exogenous folate deficiency on intracellular folate stores. These observations were confirmed in an in vivo model where GGH inhibition resulted in a significantly higher chemosensitivity in both cell lines to 5-FU at all folate concentrations [177]. Therefore, these studies demonstrate the ability of enzymes involved in the folate pathway to modulate chemosensitivity of cancer cells to 5-FU, and the complex interactions of these enzymes with exogenous folate levels in modulating 5-FU chemosensitivity.

Several genes in the folate pathway, such as TS and DHFR are known to be regulated post-transcriptionally, although more studies are needed to understand their regulatory regions and their modulation. TS can not only inhibit its own transcription by blocking transcription factors from binding to its promoter region [94], but can also exert translational control by directly binding to its own mRNA [178]. Similarly, DHFR expression is also controlled at the translational level, as DHFR can bind its own mRNA, thereby inhibiting translation [179]. Post-transcriptionally, DHFR expression may be regulated by mir-24 micro RNA, since DHFR contains a mir-24 binding site in its 3′-untranslated region [179]. In contrast, little is known about the complex regulation of ABC family drug transporters. In the past decade, numerous micro RNAs have been found to post-transcriptionally regulate the expression of MRP1, 5 and 8 in a tissue specific manner by binding to the 3′-untranslated region of the target mRNA [180]. For instance, a recent comparison of parental MCF-7 cells to doxorubicin-resistant MCF-7 cells revealed that in the resistant cell line, the expression level of mir-451 was below the detection
limit [181]. Furthermore, sensitivity to doxorubicin was restored upon transfection with mir-451 [181]. Although there is increasing knowledge on miRNA mediated post-transcriptional regulation, further studies are warranted to elucidate other translational regulatory mechanisms.

Although evidence available to date generally suggests that folate deficiency or modulation of enzymes resulting in intracellular folate depletion leads to increased chemosensitivity to 5-FU, the effects of FA supplementation on 5-FU chemosensitivity are inconsistent. In view of the high folate status of the North American population and prevalent supplement use among breast cancer patients, research is needed to clarify the current conundrum.
Table 2.4.3.1. Summary of studies that have directly examined the effect of FA deficiency on 5-FU chemosensitivity.

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Study Design</th>
<th>Folate level</th>
<th>Effect of FA deficiency on 5-FU chemosensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Backus et al., 2000</td>
<td>In vitro</td>
<td>0.5, 1.0, and 2.5 nM of FA</td>
<td>• Cells cultured at 0.5, 1.0, and 2.5 nM of FA exhibited a 2-5 fold higher chemosensitivity to 5-FU alone or in combination with LV compared to cells cultured in 8 µM FA (p&lt;0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 µM FA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tucker et al., 2002</td>
<td>In vivo</td>
<td>0, 4 or 6 ppm FA diet</td>
<td>• 0 ppm FA diet induced regression of pre-existing tumors, and inhibited post-therapeutic rebound in tumor burden (p&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• In mice on 4 and 6 ppm FA diet, treatment with 5-FU only resulted in the suppression of the expected increase in tumor burden and no regression of pre-existing tumors (p&lt;0.005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Mice on 6 ppm FA diet treated with 5-FU exhibited a tumor burden nearly identical to that in untreated age-matched controls six weeks after termination of therapy (p&lt;0.001)</td>
</tr>
<tr>
<td>Raghunathan et al., 1997</td>
<td>In vivo</td>
<td>0.01 ppm FA diet (deplete) 5.9 ppm FA diet (control)</td>
<td>• Mammary adenocarcinomas growth was suppressed approximately 80% when FU was administered to folate depleted mice 1 hr after LV administration, relative to approximately 50% suppression in control mice (p=0.001)</td>
</tr>
</tbody>
</table>
Table 2.4.3.2. Summary of studies that have directly examined the effect of FA supplementation on 5-FU chemosensitivity.

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Study design</th>
<th>Folate level</th>
<th>Effect of FA supplementation on 5-FU chemosensitivity</th>
<th>Effect of FA Supplementation on Chemosensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branda et al., 1998</td>
<td><em>In vivo</em> Weanling female Fischer 344 rats</td>
<td>low (0 ppm FA), control (2 mg/kg FA), or high FA diets</td>
<td>• Upon treatment with 5-FU, higher tumor inhibition was reported in animals receiving the high FA diet compared to the low FA diet (p=0.025)</td>
<td>↑</td>
</tr>
<tr>
<td>Raghunathan et al., 1999</td>
<td><em>In vivo</em> C3H mouse mammary adenocarcinoma</td>
<td>45 mg/kg FA was injected to folate depleted mice 4 hours before 5-FU injection</td>
<td>• 5-FU alone suppressed C3H mammary adenocarcinoma growth by 25%, whereas FA in combination with 5-FU increased growth suppression to over 70%</td>
<td>↑</td>
</tr>
</tbody>
</table>
| Ishiguro et al., 2016   | Mouse human HCT116 colon cancer xenograft model | Mice kept on control (2 mg/kg FA), 4× or 12.5× supplemental levels FA diets | • In the control group, 5-FU significantly inhibited the growth of the xenografts (P < 0.0001)  
• In the 4× supplemental FA group, 5-FU-treated xenografts grew faster than saline treated xenografts (P = 0.048)  
• In the 12.5× supplemental group, 5-FU exhibited no effect | ↓ |
2.4.4 Mechanisms of folate-induced drug resistance

There has been an increasing body of evidence regarding the role of intracellular folates in modulating 5-FU chemosensitivity. This modulatory effect of folate status is currently mainly attributed to increased TS expression and induction of MRPs.

2.4.4.1 Increased TS expression

When 5-FU is metabolized, one of its cytotoxic metabolites, 5FdUMP binds TS to form an inhibitory ternary complex, resulting in a decrease in unbound TS stores. Consequently, TS expression becomes increased since unbound TS inhibits its own transcription by blocking transcription factors from binding to its promoter region [94]. As discussed earlier, higher TS expression has been associated with decreased sensitivity to 5-FU, because an elevated concentration of TS that exceeds the inhibitory capacity of 5FdUMP would enable a proportion of the TS pool to continue to support thymidylate synthesis [182]. Consequently, TS expression is an inverse marker of 5FU efficacy, where higher concentrations of the enzyme are associated with lower 5-FU efficacy. The mechanism of TS regulation has been shown to be modulated by folate. In a high folate environment, unbound TS has been shown to dissociate from its promoter, resulting in increased transcription and increased TS stores [178]. Indeed, mathematical modeling confirms that TS continues to rise with increasing folate levels [183]. Since higher TS levels are associated with lower 5-FU efficacy, it is imperative to examine how high folate status from fortified dietary sources and FA supplementation would affect 5-FU efficacy.
2.4.4.2 Induction of MRPs

Recent evidence from *in vitro* studies suggests that high intracellular folate concentrations can lead to increased expression and activity of MRPs, resulting in increased efflux of folates, antifolates and cytotoxic metabolites such as 5FdUMP [184]. As stated in section 2.1.7.2, specifically MRP5 (ABCC5), MRP8 (ABCC11) and ABCB5 are implicated in 5-FU efflux; however ABCB5 is not expressed in breast tissue [98]. In addition to these three transporters, the study by Sadahiro and colleagues also demonstrated low MRP1 expression was predictive of high intracellular folate concentrations upon LV treatment [99]. Although MRP1 is associated with methotrexate (a prototypic antifolate) resistance, MRP1 may modulate 5-FU efficacy through mediating LV efflux [98]. Other than exporting short chain polyglutamate forms of methotrexate, MRP1 may also regulate intracellular folate stores by exporting monoglutamate or short-chain polyglutamate forms of folate such as LV, resulting in a decrease in the efficacy of LV to potentiate the cytotoxicity of 5-FU [99]. Unlike MRP1, MRP5 is involved only in the efflux of one of the active cytotoxic metabolite of 5-FU, 5FdUMP, whereas MRP8 mediates the efflux of 5-FU, 5-fluoro-2’-deoxyuridine, and 5-fluoro-5’-deoxyuridine alike [98]. At present, whether intracellular folate can modulate the induction of MRP5 and MRP8 is unknown, although MRP activity is generally expected to increase with increasing folate concentrations [98]. Currently, a generally accepted hypothesis is the inverse U-association relative to efficacy of antifolates, where at low folate concentrations, patients are adversely affected by drug toxicity, whereas at high folate concentrations, MRP activity increases, leading to drug resistance [98].
To date, the relationship between 5-FU and MRPs is poorly understood as MRPs have not only been implicated in resistance to 5-FU but also in increasing 5-FU efficacy. This dual effect appears to be modulated mostly by the total intracellular folate stores and the degree of polyglutamylation [98]. A recent model has been proposed whereby the subcellular localization of MRPs determines the effect. This model postulates that 1) when MRPs are located on the plasma membrane, FA supplementation would cause an increase in MRP expression and 2) when MRPs are located intracellularly, FA supplementation would cause a decrease in MRP expression [185]. Since 5-FU is the cornerstone of chemotherapy formulations used in the treatment of breast cancer, it is imperative to examine whether increased folate levels prevalent today would adversely affect the response to 5-FU treatment in breast cancer patients and if so, whether this effect might be mediated through the induction of MRPs.
3 Chapter 3: Problem Formulation and Research Objectives

3.1 Current state of the problem

Breast cancer patients presenting with aggressive subtypes of breast cancer rely on systemic combination chemotherapy formulations, of which 5-FU is an active component. The standard adjuvant chemotherapy formulation for breast cancer is FEC-D (5-FU, epirubicin, cyclophosphamide-docetaxal) [5, 186]. The main cytotoxicity of 5-FU in cancer cells is through the formation of an inhibitory ternary complex composed of its cytotoxic metabolite, 5FdUMP, TS and 5,10-methyleneTHF, thereby inhibiting TS and DNA synthesis [8]. Since TS and 5,10-methyleneTHF are an important enzyme and metabolite, respectively, in the folate metabolic and one-carbon transfer pathways, the cytotoxicity of 5-FU depends in part on the disruption of folate metabolism.

Folate is critical for maintaining human health as it mediates biological one-carbon transfer reactions involved in nucleotide biosynthesis and biological methylation reactions [9]. Based on a convincing body of evidence for a protective effect of periconceptional FA supplementation on the risk of NTDs, mandatory FA fortification of white wheat flour was implemented in 53 countries, including the US and Canada [13-15]. Additionally, the widespread use of dietary supplements has resulted in approximately 30-40% of the North American population consuming multivitamins that usually contain 400 µg of FA [16-19]. Studies have demonstrated that multivitamin use is even higher in cancer patients and survivors, as 14-32% cancer patients start using supplements after diagnosis [22]. Since female
gender is usually associated with supplement use, it is not surprising that of all cancer patients, breast cancer patients were found to have the highest prevalence of multivitamin use at 57-62% [22]. At present, FA fortification of the food supply combined with widespread supplement use have resulted in significantly higher intakes and blood levels of folate and FA in North America compared with pre-FA fortification era [20, 21]. The most recent Canadian Health Measures Survey reported that >40% of the Canadian population had red blood cell (RBC) folate concentrations above an arbitrary high concentration cut-off (defined as levels above the 97th percentile of RBC folate concentrations from NHANES 1999-2004 at 1360 nmol/L) [21].

High intakes and blood levels of folate and FA from dietary fortification and prevalent supplement use in breast cancer patients are concerning, given emerging evidence that suggests a tumor promoting effect of high folate status, primarily from FA supplementation. Although the relationship between folate status and breast cancer risk has not been clearly established, recent epidemiologic studies have reported an increased risk of breast cancer with high folate levels [23-28]. Furthermore, two recent animal studies suggest that FA supplementation increases mammary tumor risk [29, 126]. Several biologically plausible mechanisms do exist to explain the tumor promoting effect of FA supplementation including: 1) increased nucleotide substrates for DNA synthesis, resulting in accelerated tumor growth; and 2) inactivation of tumor suppressor genes through de novo methylation of promoter CpG islands of tumor suppressor genes [150].

At present, the role of folate in modulating 5-FU sensitivity has not been well established. Few in vitro and in vivo studies have reported that folate deficiency increases
chemosensitivity of cancer cells to 5-FU [32-34]. Conversely, two in vivo studies have suggested that FA supplementation can increase sensitivity of breast cancer cells to 5-FU [35, 36], whereas another in vivo study conducted in a mouse human HCT116 colon cancer xenograft model, has demonstrated that FA supplementation decreases the efficacy of 5-FU treatment [37]. One of the in vivo breast cancer studies only captured the effect of short-term FA supplementation (FA co-administered with 5-FU to folate deficient mice) as opposed to chronic long-term supplementation prevalent in the breast cancer population today. Additionally, in vivo xenograft models have several limitations such as the necessity to use immunocompromised mice [187]. The absence of a functional immune system will significantly impact tumor progression and development [187]. In order for xenografts to be established, human breast cancer cell lines are injected subcutaneously into the flank or into the mammary fat pad of the animal, however both these approaches significantly alter the growth and metastatic potential of these cells because in humans the epithelial and stromal compartments co-evolve during carcinogenesis [187].

In summary, there is a paucity of studies that examine whether FA supplementation would influence chemosensitivity of breast cancer cells to 5-FU at present. Furthermore, potential mechanisms by which FA supplementation may affect 5-FU chemosensitivity have not been clearly elucidated. Given the drastically increased intakes and blood levels of folate and FA in the Canadian population, in particular those afflicted with breast cancer, whether or not FA supplementation would adversely affect 5-FU-based chemotherapy for breast cancer is an important health issue.
3.2 Research rationale

Given the high incidence of breast cancer in women worldwide, widespread supplement use by 67-87% of breast cancer patients, and the prevalence of 5-FU-based chemotherapy treatments in breast cancer patients, it is important to examine whether chemosensitivity of breast cancer cells to 5-FU is reduced by FA supplementation. Folate deficiency has been shown to increase chemosensitivity of cancer cells to 5-FU in vitro [32] and in animal studies [33, 34] but the effect of FA supplementation on 5-FU chemosensitivity has not been well established. Potential mechanisms by which FA supplementation may modulate chemosensitivity of human cancer cells to 5-FU are largely unknown at present. There are no studies, to date, that have evaluated whether FA supplementation influences chemosensitivity of breast cancer cells to 5-FU in an in vitro model. It is imperative to elucidate optimal FA levels in breast cancer patients during treatment with 5-FU-based chemotherapy, to maximize the efficacy of the treatment and minimize toxicities and side effects. Given these considerations, my research aimed to investigate the effect of FA supplementation on chemosensitivity of human breast cancer cells to 5-FU in an in vitro model and to elucidate potential mechanisms underlying this effect.

3.3 Research hypothesis

Human breast cancer cells grown in vitro in medium containing 500 and 1000 nM FA (chosen to approximate supplemental folate levels of 10x and 20x the mean serum folate levels in the population) will exhibit decreased chemosensitivity to 5-FU compared to cells grown in 5 nM FA (deficiency) and 50 nM FA (mean serum folate concentrations in North America).
3.4 Research objectives

1. To investigate if FA supplementation modulates chemosensitivity of human MCF-7, MDA-MB-231 and HCC1937 breast cancer cell lines to 5-FU in an in vitro model.

2. To elucidate potential mechanisms underlying the modulatory effects of FA supplementation on chemosensitivity of human breast cancer cells to 5-FU by:
   a. Investigating changes in gene expression of enzymes involved in folate metabolism and DNA synthesis such as TS, DHFR, and MTHFR.
   b. Investigating changes in gene expression of transporters responsible for the efflux of folate and active metabolites of 5-FU such as MRP1, 5 and 8.
Chapter 4: The effect of folic acid supplementation on chemosensitivity of human breast cancer cells to 5-fluorouracil in vitro

4.1 Introduction

Folate plays a critical role in de novo nucleotide biosynthesis and biological methylation reactions. In the past two decades, mandatory FA fortification of the food supply, coupled with prevalent supplement use have resulted in dramatically increased intakes and blood levels of folate and FA in the North American population [20, 21]. In particular, 67-87% of breast cancer patients report using multivitamins containing FA after diagnosis [22]. Concerns have been raised whether high intake and blood levels of folate and FA may increase breast cancer risk by promoting (pre)neoplastic lesions in the breast tissue [23-29, 126]. Furthermore, biologically plausible mechanisms exist to suggest that high folate status may adversely affect 5-FU chemotherapy in breast cancer patients. 5-FU is a cornerstone in chemotherapy formulations used in the treatment of breast cancer [5, 186]. Indeed, a recent in vivo study has shown that FA supplementation may increase resistance of human colon cancer cells to 5-FU [37]. However, whether or not FA supplementation can diminish the response of human breast cancer cells to 5-FU is unknown at present. Therefore, this study aims to investigate whether FA supplementation can affect chemosensitivity of human breast cancer cells to 5-FU chemotherapy in an in vitro model and to elucidate potential mechanisms underlying this effect.
4.2 Materials and Methods

4.2.1 Cell lines

Since the luminal A subtype of breast cancer has the highest incidence, accounting for 40% of all breast cancer cases diagnosed, the MCF-7 cell line (Luminal A: ER+/PR+/HER2-/Ki-67 low) [188] was chosen to model this prevalent subtype of breast cancer. Unlike the other subtypes, TNBC patients present with a more aggressive phenotype, have no targeted treatment options and rely on chemotherapy for improved survival [4, 7]. As TNBC patients have poor prognosis and their survival is more dependent on chemotherapy treatment, two TNBC cancer cell lines were chosen to investigate this hypothesis: MDA-MB-231 (ER-/PR-/HER2-) [188] and HCC1937 (ER-/PR-/HER2- and homozygous BRCA1 5382C mutation) [189]. By using these three human breast cancer cell lines, we aimed to examine differences in proliferation, chemosensitivity and gene expression among luminal A, basal-like breast TNBC and hereditary TNBC modeled by MCF-7, MDA-MB-231 and HCC1937 cell lines, respectively. Phenotypic and molecular differences of the selected cell lines are summarized in Table 4.2.1.

The MCF-7 and MDA-MB-231 cell lines were generously provided by Dr. Lilian Thompson’s research group (Department of Nutritional Sciences, University of Toronto) and were two and three passages from American Type Culture Collection (Manassas, VA, USA) authentication, respectively. The HCC1937 cell line was obtained directly from the American Type Culture Collection (Manassas, VA, USA).
Table 4.2.1. Phenotypic and molecular characteristics of HCC1937, MCF-7 and MDA-MB-231 human breast cancer cell lines.

<table>
<thead>
<tr>
<th></th>
<th>HCC1937 [190]</th>
<th>MCF-7 [188]</th>
<th>MDA-MB-231 [188]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td>Epithelial</td>
<td>Epithelial</td>
<td>Epithelial</td>
</tr>
<tr>
<td><strong>Growth Properties</strong></td>
<td>Adherent</td>
<td>Adherent</td>
<td>Adherent</td>
</tr>
<tr>
<td><strong>Doubling time</strong></td>
<td>60</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td><strong>Chromosome Number</strong></td>
<td>100 (modal chromosome number)</td>
<td>82 (modal chromosome number)</td>
<td>64 (modal chromosome number)</td>
</tr>
<tr>
<td><strong>Receptor Features</strong></td>
<td>ER-/PR-/HER2-</td>
<td>ER+/PR+/HER2-</td>
<td>ER-/PR-/HER2-</td>
</tr>
<tr>
<td><strong>Molecular Features</strong></td>
<td>BRCA1 (mutated, insertion C at nucleotide 5382), p53-, PTEN -, Epithelial glycoprotein 2+, cytokeratin 19+</td>
<td>WNT7B oncogene expressed, insulin-like growth factor binding proteins: BP-2, BP-4, BP-5 expressed</td>
<td>Epidermal growth factor expressed, transforming growth factor alpha expressed, WNT7B oncogene expressed</td>
</tr>
</tbody>
</table>

4.2.2 Cell culture

All three cell lines were cultured in RPMI-1640 FA-free medium (Invitrogen, Gaithersburg, MD, USA) supplemented with appropriate amounts of standard RPMI-1640 medium (2.3 µmol/L FA) to yield concentrations of 5, 50, 500 and 1000 nmol/L FA, respectively. Medium FA concentrations were not measured to confirm concentrations, which is a limitation of this study. Growth medium was supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin, and 0.1% fungizone. Cells were maintained at 37°C in 95% humidity and 5% CO₂ and passaged at approximately 80-90% confluency.

4.2.3 Growth curves

Two million cells were counted and seeded in triplicate in 100 mm plates containing 5 nM, 50 nM, 500 nM or 1000 nM FA RPMI-1640 medium. 5 nM FA was chosen to parallel deficiency, 50 nM was chosen to parallel the mean serum folate concentrations in North
America [20], and 500 and 1000 nM FA were chosen to approximate supplemental folate levels of 10x and 20x the mean serum folate levels in the population. Three 100 mm plates were seeded for each folate concentration to yield 12 plates for each cell line to be harvested at each time point. Each biological replicate was counted three times (technical replicates) and the mean of the technical replicates was used as the value for each biological replicate. Consequently, values at each time point were derived from 3 biological replicates to yield a sample size of 3 for subsequent statistical analysis. Cells were counted and passaged at the same splitting ratio of 2:3 every four days up to fifteen days, using the Vi-CELL Series Cell Viability Analyzer (Beckman Coulter) that provides accurate and precise total cell number and viability by trypan blue staining.

### 4.2.4 Determinants of intracellular folate concentrations

Intracellular folate concentrations were determined using a standard microbiological *Lactobacillus rhamnosus* (ATCC# 7469, Manassas, VA) microtiter plate method [191]. At each folate treatment group, 10x10⁶ cells were trypsinized, washed twice with PBS and stored at -80°C in 1% ascorbic acid with PBS until the day of the folate assay. On the day of the intracellular folate determination, cells were thawed, sonicated 3 times for 15 seconds, while being kept on ice. Cell lysates were diluted with equal volumes of 0.1M K₂HPO₄ and were incubated with chicken pancreas conjugase for 2 hours at 37°C. Conjugase converts all polyglutamylted folates into their corresponding diglutamate derivatives, which allow for total intracellular folate determination since *L. rhamnosus* grows equally well at mono, di and triglutamyl folate forms [191]. Aliquots of cell lysate were incubated in duplicate with *L. rhamnosus* in mylar sealed 96 well plates overnight for approximately 16 hours. Since *L.
*rhamnosus* bacteria grow proportionally to the amount of folate present, turbidity was measured spectrophotometrically at a wavelength of 650 nm and using a standard curve of known folate concentrations, intracellular folate concentrations in the sample were calculated.

### 4.2.4.1 Folic acid standard preparation

Ten milligrams FA was dissolved in 10 mL of ddH2O with 5 µL 10 M NaOH to 1 mg FA/mL. pH was adjusted to pH 7 to 8 with HCl and the concentration was verified by spectrophotometry (280 nm). The solution was diluted to 2 ng/mL with 0.1 M KPO4 buffer (1.05 g KH2PO4, 0.4 g K2HPO4, 0.1 g Na ascorbate, 100 mL ddH2O, filter sterilized), aliquoted, and stored at -80°C.

### 4.2.4.2 Lactobacillus rhamnosus stock preparation

Two hundred microliters *L. rhamnosus* (ATCC# 7469, Manassas, VA) stock was incubated with Lactobacillus MRS broth for 18 hours at 37°C. Under aseptic conditions, cells were centrifuged and the supernatant was decanted. Cell pellet was resuspended in 180 mL of MRS broth and 20 mL of cold 100% glycerol. Solution was aliquoted and stored at -80°C.

### 4.2.4.3 Chicken pancreas conjugase preparation

Chicken pancreas acetone powder (Difco, 0459-12-12) was dissolved in 0.1 M KPO4 buffer (Table 4.3.4.4) and incubated for 6 hours at 37°C under a blanket of toluene. Toluene was removed and the solution was centrifuged at 10 000 x g for 15 minutes. The supernatant was collected and added to an equal volume of tricalcium phosphate (BioRad Get HTP was rehydrated: 1 part HTP to 6 parts 0.1 M KPO4 buffer, per 10g HTP). The solution was stirred for 30 minutes at 4°C and centrifuged at 10 000 x g for 30 minutes at 4°C. The supernatant was
cooled to 4°C, added to an equal volume of 95% ethanol, and left overnight at -20°C. The day after, the solution was centrifuged at 10 000 x g for 30 minutes, supernatant was removed, and resuspended in 50 mL of cold 1.0 M KPO₄ buffer. Ten grams of Dowex-1 (BioRad AG1-X8) was added, stirred for 1 hour at 4°C, and filtered at 4°C. The solution was aliquoted and stored at -80°C.

4.2.4.4 Intracellular folate determination

Three µL of *L. rhamnosus* stock was inoculated in 3 mL Lactobacillus MRS broth and incubated in a shaker at 37°C for 16-18 hours. The following day, 500 µL of culture were reinoculated in 2.5 mL Lactobacillus MRS broth and further incubated in a shaker at 37°C for 6 hours. Bacterial growth, or turbidity, was confirmed by optical density as measured by spectrophotometer at 650 nm (O.D. of 1.8 is considered optimal, but up to 2 is acceptable).

Fresh 0.1 M KPO₄ buffer and FA media were prepared (quantity of each was dependent on the number of 96 well plates to be used), the composition of both is indicated in Table 4.2.4.1. 150 µL of 0.1 M KPO₄ buffer was added to each well in a clear 96-well microtiter plate. Samples were then added to specified wells in duplicate (up to 150 µL for cell lysates grown in low FA medium). If less than 150 µL of cell lysate was added, 0.1 M KPO₄ buffer was added to the same wells to bring up the total well volume to 300 µL. These wells were serially diluted three times resulting in four measurements per sample. The folate standard stock was diluted to 2 ng/mL in 0.1 M KPO₄ buffer, added to two specified wells in duplicate and these wells were serially diluted seven times resulting in 8 measurements that were used to generate the standard curve.
Under aseptic conditions, *L. rhamnosus* inoculum was centrifuged at 5000 rpm for 5 minutes to sediment bacteria. Supernatant was decanted and bacteria was resuspended in 3 mL sterile folic acid medium (Table 4.2.4.1), and centrifuged at 5000 rpm for 5 minutes. This washing step was critical to eliminate residual folate from the MRS broth and was repeated four more times. After the final resuspension, the inoculum mixture was diluted with folic acid media (24x), and further diluted (40x). 150 μL of the 40x diluted inoculum was added to each well of the plate, to bring the final volume up to 300 μL. Plates were covered with mylar sealers and incubated at 37°C for 16-18 hours. Plates were read spectrophotometrically at a wavelength of 650 nm and using a standard curve of known folate concentrations, intracellular folate concentrations were calculated in the sample using SoftMax software.

Table 4.2.4.1. Composition of 0.1 M KPO₄ buffer and folic acid media for folate assay.

<table>
<thead>
<tr>
<th>0.1 M KPO₄ buffer</th>
<th>Folic Acid Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mL needed per plate</td>
<td>20 mL needed per plate + 40 mL for washing</td>
</tr>
<tr>
<td>1.05 g KH₂PO₄</td>
<td>9.4 g folic acid media</td>
</tr>
<tr>
<td>0.4 g K₂HPO₄</td>
<td>0.05 g Na ascorbate</td>
</tr>
<tr>
<td>0.1 g Na ascorbate</td>
<td>ddH₂O (up to 100 mL)</td>
</tr>
<tr>
<td>ddH₂O (up to 100 mL)</td>
<td>Boil solution for 2 minutes to dissolve, allow to cool and sterilize by vacuum filtration.</td>
</tr>
<tr>
<td>Stir solution with a magnetic stirrer and sterilize by vacuum filtration.</td>
<td></td>
</tr>
</tbody>
</table>

4.2.5 Drug preparation

Both LV and 5-FU solutions were prepared fresh within 30 minutes of application on the second day of the *in vitro* chemosensitivity assay. To make 5-FU (Sigma, F6627, liquid) dilutions for each of the 4 concentrations of FA, 1 mL of 10 mg 5-FU liquid solution was added to 538 μL of corresponding medium to make 0.05 M 5-FU stock. This was further diluted 1 to 50, meaning 60 μL of 0.05 M 5-FU was added to 2940 μL of corresponding medium to yield a 5 x
Eight 15 mL Falcon tubes were filled with 10 mL of respective FA media, labeled 1 through 8, resulting in 32 tubes [(8 tubes times 4 concentrations of FA(5, 50, 500, 1000 nM)]]. The final concentrations of 5-FU ranged from 25 µM (tube 1) to 1.5 µM (tube 8) for each concentration of FA. These final concentrations were achieved by adding the volume of 5 x 10⁻³ M 5-FU stock to each tube as indicated in Table 4.2.5.

Table 4.2.5. Preparation of 5-FU for *in vitro* chemosensitivity.

<table>
<thead>
<tr>
<th>Final Concentration of 5-FU (µM)</th>
<th>Volume of 5 x 10⁻³ M 5-FU to be added (µL) per 10 mL final volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) 25</td>
<td>500</td>
</tr>
<tr>
<td>2) 20</td>
<td>400</td>
</tr>
<tr>
<td>3) 15</td>
<td>300</td>
</tr>
<tr>
<td>4) 10</td>
<td>200</td>
</tr>
<tr>
<td>5) 7.5</td>
<td>150</td>
</tr>
<tr>
<td>6) 5</td>
<td>100</td>
</tr>
<tr>
<td>7) 3</td>
<td>60</td>
</tr>
<tr>
<td>8) 1.5</td>
<td>30</td>
</tr>
</tbody>
</table>

To dilute LV to a final concentration of 5 µM in each well, 0.005 g of LV (Sigma, F7878, powder) was dissolved in 1 mL of ddH₂O and 3 µL of 2 M NaOH, to a concentration of 10 mM. 5 µL of the 10 mM concentration was added to each of the 32 tubes that contained 5 mL after treatment with 5-FU alone, resulting in an LV concentration of 10 µM. When 100 µL of the 10 µM was added to each well containing 100 µL of medium from day one, the concentration of LV became 5 µM in each well.

4.2.6 *In vitro* chemosensitivity

*In vitro* chemosensitivity was determined using a modified Sulforhodamine B (SRB) protein assay as previously described [192, 193]. The modified SRB protein assays have been extensively used in previous *in vitro* 5-FU chemosensitivity studies [175-177]. 8000 cells per
100 μL of respective FA concentration in RPMI-1640 medium were seeded in duplicate in 96-well flat-bottom plates (Figure 4.3.6). After 24 hours, an additional 100 μl of corresponding FA medium containing 5-FU (InvivoGen, San Diego, CA, USA) and LV (Sigma) were added, and cells were cultured for an additional 72 hours. As stated previously, medium FA concentrations were 5, 50, 500 and 1000 nmol/L FA. In all three cell lines, concentrations of 5FU ranged from 1.5 μM to 25 μM, whereas the concentration of LV was held constant at 5 μM.

The pharmacokinetic properties of 5-FU vary significantly not only at the interpatient level but also at the intra-patient level [194]. Mode of administration (oral vs. intravenous) and DPD expression also significantly affect serum and tissue 5-FU levels [194]. Currently, there are no data on intracellular 5-FU metabolite levels in neither non-transformed mammary cells nor cancer cells. In 2015, Derissen et al developed a highly sensitive LC-MS/MS assay for the quantitative determination of the intracellular 5-FU nucleotides [195]. This technique allowed for intracellular 5-FU metabolite 5FUTP, 5FdUTP and 5FdUMP levels to be measured. Only FUTP could be measured in the peripheral blood mononuclear cells, ranging from 1.0 μM on day 1 to 14 μM on day 14 [196]. FdUTP and FdUMP intracellular concentrations could not be measured as they were below the detection limits [196].

Current doses of 5-FU in chemotherapy formulations used for the treatment of breast cancer range from 500 to 600 mg/m² coupled with a fixed dose of LV at 100 mg/m² [4, 5]. Due to the variability in 5-FU dose, treatment duration and differential expression of DPD prior to 5-FU uptake into peripheral breast tissue, it is difficult to mimic the clinical setting [194]. The range of 5-FU dose used in our model (1.5 μM to 25 μM) falls within the range of intracellular 5-FU metabolite levels reported by Derissen et al and captures a wide range in percent cell
survival, from 50-90% in HCC1937 cells, 25-70% in MCF-7 cells and 30-80% in MDA-MB-231 cells. Since the highest dose of 25 µM was lower than the standard 500 mg/m² by a factor of 20,000, the same factor was applied to the clinically fixed dose of LV at 100 mg/m² to yield a fixed dose of 5 µM. The selected 5-FU and LV concentrations were previously used by our group to mimic the clinical setting for the treatment of breast cancer [175, 176].

![Schematic depicting the five day in vitro chemosensitivity assay using Sulforhodamine B (SRB) protein dye](image)

**Day 1**
Plate 8000 cells/well

<table>
<thead>
<tr>
<th>FA Concentration (nM)</th>
<th>Plate 1</th>
<th>Plate 2</th>
<th>Plate 3</th>
<th>Plate 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 nM control</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>50 nM control</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>500 nM control</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>1000 nM control</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

**Day 2**
Fix control plates

**Day 3**

**Day 4**

**Day 5**
Fix test plates and stain with SRB protein dye and read OD at 570 nm.

**Survival (%)** = \[\frac{\text{OD}_{\text{drug}}}{\text{OD}_{\text{start drug exposure}}} - 1\] \[\div\] \[\frac{\text{OD}_{\text{no drug}}}{\text{OD}_{\text{start drug exposure}}} - 1\] \[\times\] 100

**Figure 4.2.6. Schematic depicting the five day in vitro chemosensitivity assay using Sulforhodamine B (SRB) protein dye.** On day one, eight plates were seeded at a density of 8000 cells/well in 100 µL of 5, 50, 500 or 1000 nM FA. Two plates were seeded per concentration of FA; on day two, one plate of each FA concentration was fixed to be used as control for start of drug exposure (shown in red rectangle). On day two, test plates were treated with 5-FU only (columns 1-5), 5-FU with fixed dose of LV at 5 µM (columns 5-10) and fresh medium corresponding to correct FA concentration without drug (columns 10-12) as a control for no drug exposure. Concentration of 5-FU decreased down rows as follows: 25 µM in row A, 20 µM in row B, 15 µM in row C, 10 µM in row D, 7.5 µM in row E, 5 µM in row F, 3 µM in row G and 1.5 µM in row H. On day 5, test plates were also fixed with TCA. All eight plates were stained with SRB, then solubilised and the OD was read at 570 nm. Results were expressed as percent cell survival based on the indicated formula.
As illustrated in Figure 4.2.6, after 72 hours, cells were fixed with trichloroacetic acid and stained with SRB protein dye. The dye was then solubilised and the OD of the solution was measured at 570 nm. The results were expressed as the percentage of cell survival on the basis of the difference between the OD at the start and end of drug exposure, according to the formula previously used and illustrated in figure 4.2.6 [197].

4.2.7 Semi-quantitative real time reverse transcriptase-PCR

4.2.7.1 Total RNA extraction

The RNeasy Microarray Tissue Mini Kit (Qiagen, catalogue no. 73304) was used to isolate RNA. First, MCF-7, HCC1937 and MDA-MB-231 cells were grown in 100 mm round flat bottomed plates at four different FA concentrations of 5, 50, 500 and 1000 nM for 5 days. On day 5, confluent plates at each concentration of FA were washed three times with 10 mL of PBS and 1 mL of QIAzol lysis reagent (Qiagen, catalogue no. 79306) was added. Cells were scraped off the plate using a cell scraper and were placed into an Eppendorf tube. Lysates were incubated at room temperature for 5 minutes to allow the dissociation of nucleoprotein complexes. 200 µL of chloroform was added to each tube, which was then shaken vigorously, followed by a 3 minute incubation period at room temperature, and centrifugation at 12, 000 x g for 15 minutes at 4°C.

The upper, aqueous phase was collected and combined with an equal volume of 70% cold ethanol. These solutions were added to an RNeasy Mini spin column, centrifuged briefly at room temperature, and eluate was discarded. In order to efficiently remove all DNA, on-column DNA digestion was performed using an RNase-free DNase set (Qiagen, catalogue no. 83
79254). First, 350 µL Buffer RW1 was added to the spin column, samples were centrifuged briefly at room temperature, and eluate was discarded. In a separate tube, 10 µL DNase I stock solution and 70 µL Buffer RDD was mixed. 80 µL of the DNase 1 stock solution was added to the spin column, and samples were placed on the benchtop for 15 minutes. Following this incubation, 350 µL of Buffer RW1 was added to the spin column again, samples were centrifuged briefly at room temperature, and eluate was discarded.

To wash the membrane, 500 µL Buffer RPE was added directly to the spin column, centrifuged briefly at room temperature, and eluate was discarded. This step was repeated again. Finally, to elute RNA, the spin column was placed in a new tube, 30 µL RNase-free water was added, and centrifuged for 1 minute at room temperature, twice. Purity and integrity of the RNA was determined using the Agilent 2100 Bioanalyzer, and RNA concentrations obtained from the bioanalyzer were used in subsequent calculations for cDNA synthesis. Extracted RNA was aliquoted and stored at -80°C until further use.

4.2.7.2 Synthesis of cDNA

The QuantiTect Reverse Transcription Kit (Qiagen, catalogue no. 205311) was used for cDNA synthesis. This kit effectively removes contaminating genomic DNA, denatures active enzymes and generates cDNA using reverse transcription. Components of the kit were thawed, centrifuged and kept on ice. 2 µL gDNA wipeout buffer (7x), 2 µL RNase-free water and 10 µL template RNA (150ng/µL) was combined in a 0.2 mL thin wall PCR tube, incubated at 42°C for 2 minutes, and immediately placed on ice. Reverse transcription master mix was prepared as follows: 4 µL Quantscript RT Buffer (5x), 1 µL RT Primer Mix, 1 µL Quantscript Reverse Transcriptase and 14 µL denatured template RNA. Samples were incubated at 42°C for 30
minutes then at 95°C for 5 minutes in order to inactivate reverse transcriptase. Newly generated cDNA was immediately placed on ice and stored at -20°C until further use.

**4.2.7.3 Semi-quantitative real time RT-PCR**

Semi-quantitative real time RT-PCR was performed using the ViiA7™ Real-Time PCR System (Applied Biosystems, Life Technologies) according to the manufacturer’s instructions. Reactions were performed using 384 well plates and a final reaction volume of 10 µL was used consisting of 0.1-0.5 µL primers with concentrations ranging from 10 to 50 µM, 5 µL Fast SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies), 2 µL cDNA template and enough RNase-free water to raise the volume to 10 µL. The primer sequences in the reaction mixtures are listed in Table 4.2.7.3. Cycling conditions were as follows: 2 minutes at 50°C, then 10 minutes at 95°C, followed by 40 cycles with 15 second denaturation at 95°C, 1 minute primer annealing at 60°C, 15 seconds of fragment elongation at 95°C. Samples were run in triplicate on each plate, and repeated at least twice on separate days.

Relative gene expression was measured using the delta delta Ct method (ΔΔCt). This method was chosen over absolute quantification because we were interested in the changes in gene expression on day five of FA treatment relative to day zero. First, Ct values across experiments were compared per sample, and outliers were removed to limit standard deviations to < 0.5. The ΔΔCt method requires an internal control to normalize the number of reactions for the amount of cDNA added to the reaction. Through primer validation, a suitable housekeeping gene, GUSB, and a calibrator (day zero samples) were chosen. Therefore, the data using the ΔΔCt method represents the fold change in gene expression normalized to an endogenous reference gene (GUSB) relative to baseline (day zero) samples for each cell line.
Gene expression data were analyzed using the ViiA7TM Real-Time PCR System (Applied Biosystems, Life Technologies).

Table 4.2.7.3. Primer sequences used for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Housekeeping Gene</strong></td>
<td></td>
</tr>
</tbody>
</table>
| GUSB (Integrated DNA Technologies, RefSeq #NM_000181) | Sense: 5'-GCC CAT TAT TCA GAG CGA GTA-3'  
|                  | Antisense: 5'-GTT TTT TCA CCA GAC CCA GAT G-3'         |
| **Investigative Genes** |                                                      |
| TS (Integrated DNA Technologies, RefSeq #NM_001071.2) | Sense: 5’-CAGCTCAGTTTCACCACACAG-3’  
|                  | Antisense: 5’-GAAATTCATCATGTCGAGCTG-3’                |
| DHFR              | Sense: 5’-ACCTGGTTTCCATCTTGAG-3’                      
|                  | Antisense: 5’-CCTGAGAGATGGTTTTCCTGAGT-3’              |
| MTHFR (Integrated DNA Technologies, RefSeq #NM_005957.4) | Sense: 5’-CGCTTCCAACAGATGCA-3’  
|                  | Antisense: 5’-CTGGCCCTGAAGAACATCA-3’                 |
| MRP1 (Integrated DNA Technologies, RefSeq #NM_004996) | Sense: 5’-TGCCATTCTCCATTTGCTTGT-3’  
|                  | Antisense: 5’-CTGAGTTCCTGGTGACCTATG-3’               |
| MRP5 (Integrated DNA Technologies, RefSeq #NM_005688) | Sense: 5’-CCTCCAGATAACTCCACCAG-3’  
|                  | Antisense: 5’-GAAGATATCTTACAGATCAACC-3’              |
| MRP8 (Integrated DNA Technologies, RefSeq #NM_000352) | Sense: 5’-CTCTTCTCCTTCTTCTTCTA-3’  
|                  | Antisense: 5’-AGACCTCATGAACGACA-3’                   |

**4.2.8 Statistical Analyses**

Differences in proliferation and means of intracellular folate concentrations between the four groups of FA were determined using one-way ANOVA in all three cell lines. If a significant interaction (alpha level p≤0.05) was observed, Tukey’s post-hoc test was performed.

For *in vitro* chemosensitivity, all experiments were performed in quintuplets and repeated at
least in three different experiments. Chemosensitivity results were representative data from three experiments, where results were consistent between experiments and trends were reproducible. In order to ascertain whether the relationship between drug dose and percent survival varied with concentration of folate, analysis of covariance (ANCOVA), more specifically ordinary least squares regression, was used to model percent survival in all three cell lines. The initial regression model for percent survival included date of the experiment, LV, folate concentration and log(5-FU) as independent terms, as well as the three way interaction between folate, LV and log(5-FU), two-way interaction between folate and LV, two-way interaction between folate and log(5-FU) and two-way interaction between LV and log (5-FU). If the interactions were found not to be significant, they were dropped from the model, and the test was re-run. The validity of the results obtained from the least squares regressions were assessed graphically by plotting the residuals vs. the predicted values and generating a quantile plot of the residuals. Once the residuals vs. the predicted values plot exhibited no pattern and constant variance, and the quantile plot appeared linear, parameter estimates were obtained for all three cell lines. One-way ANOVA, followed by Tukey’s post-hoc multiple comparisons test was used to compare gene expression differences between groups. Statistical tests were performed using SAS University Edition (SAS Institute, Cary, NC, USA) and GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla, California, USA). Similarly, graphs for in vitro chemosensitivity were prepared in Excel, and all other graphs were prepared through Graphpad Prism. All statistical tests were two-sided and considered significant at alpha level p≤0.05.
4.3 Results

4.3.1 Growth curves

In all 3 cell lines, cells cultured at 5 nM FA (deficiency model) exhibited slower growth rates compared with those cultured at 50, 500 and 1000 nM FA (p<0.0001) (Figure 4.3.1). FA supplementation at 1000 nM was associated with higher growth rates than 50 nM in the HCC1937 (p=0.0161) and MDA-MB-231 (p=0.0032) cell lines but not in the MCF-7 cell line. In contrast, FA supplementation at 500 nM was associated with a higher growth rate than 50 nM only in the HCC1937 cell line (p=0.0434). No difference in growth rates was observed between 500 nM and 1000 nM in the HCC1937 and MCF-7 cell lines but in the MDA-MB-231 cell line, FA supplementation at 1000 nM was associated with a higher growth rate than 500 nM (p=0.0032). Therefore, growth rates in response to FA supplementation were cell-specific and no consistent dose-responsive relationship was observed (Figure 4.3.1.).
Figure 4.3.1. Fifteen day growth curve of (A) HCC1937, (B) MCF-7 and (C) MDA-MB-231 cell lines cultured in 5, 50, 500 and 1000 nM FA RPMI 1640 medium. Cells were counted and passaged on days 4, 8, and 12 up to day fifteen. In all 3 cell lines, cells cultured at 5 nM FA exhibited slower growth rates compared with those cultured at 50, 500 and 1000 nM FA (p<0.0001). Generally, growth rates in response to FA supplementation were cell-specific and a clear dose-responsive relationship was not observed. Means significantly different among medium FA groups within the same time point are denoted with an asterisk at p<0.05. Results expressed as mean ± SD.
4.3.2 Intracellular folate concentrations

Intracellular folate concentrations were significantly different among cells cultured in 4 different FA levels in all 3 cell lines (p<0.05) (Figure 4.3.2). In the HCC1936 cell line, a clear dose-responsive relationship was observed between intracellular folate concentrations and FA concentrations in the medium (p≤ 0.0003). In the MCF-7 cell line, intracellular folate concentrations generally reflected FA concentrations in the medium but they appear to be plateaued at 500 nM medium FA concentration. In the MDA-MB-231, a dose-responsive relationship was observed between intracellular folate concentrations and medium FA levels at day 5 but at day 10 and day 15, intracellular folate concentrations decreased with 1000 nM FA in the medium. Interestingly, in all three cell lines, intracellular folate concentrations seem to decrease over time at each medium FA concentration in a cell-specific and medium FA concentration-dependent manner (p≤0.0073) (Figure 4.3.2).

Of particular interest were intracellular folate concentrations at day five because the in vitro chemosensitivity assay previously described is five days long, and it was critical to confirm whether five days were sufficient to alter intracellular folate concentrations that may in turn affect chemosensitivity. Figure 4.3.2 panel A shows on day five, intracellular folate concentrations were significantly different among cells cultured in 5, 50, 500 and 1000 nM FA levels in all 3 cell lines (p ≤ 0.0364 in MCF-7 cells; p ≤ 0.0003 in HCC1937 cells; p < 0.0001 in MDA-MB- 231 cells).
Figure 4.3.2. Intracellular folate concentrations of HCC 1937, MCF-7 and MDA-MB-231 cells cultured in 5, 50, 500 and 1000 nM FA medium concentration at days 0, 5, 10 and 15. Intracellular folate concentrations were determined using a standard microtiter plate assay using *Lactobacillus rhamnosus* following cellular folate extraction and treatment with chicken pancreas conjugase. In panel A means statistically significant among different medium FA groups within the same time point are denoted with different letters at p<0.05 for each cell line. In panel B, means statistically significant at different time points (left to right: day 0, 5, 10, 15) in the same medium FA concentration are denoted with different letters at p<0.05 for each cell line. Results expressed as mean ± SD.
4.3.3 *In vitro* chemosensitivity to 5-FU

4.3.3.1 HCC1937 chemosensitivity to 5-FU

Figure. 4.3.3.1.1 Predicted percent survival for HCC1937 cells based on final regression model: percent survival = (date) (FA) (LV) (log 5-FU) (FA*LV) (log 5-FU*LV), where * indicates an interaction. Panels A and B depict predicted percent survival for HCC1937 cells cultured without LV and with LV, respectively.
Figure. 4.3.3.1.2 Comparison between predicted percent survival for HCC1937 cells based on final regression model treated with 5-FU alone or 5-FU with the addition of LV among cells grown in different FA medium concentrations. Panels (A-D) depict HCC1937 cells grown in 5 nM, 50 nM, 500 nM and 1000 nM FA medium respectively, where solid and dashed lines represent percent survival with and without the addition of LV, respectively. * Indicates statistically significant difference at p<0.05.

In the HCC1937 cells, the three way interaction between FA, LV and \( \log_{10} (5\text{-FU}) \) and two-way interaction between \( \log_{10} (5\text{-FU}) \) and FA were not significant (p=0.84 and p=0.34, respectively) and were dropped from the model. The two-way interactions between FA and LV and between \( \log_{10} (5\text{-FU}) \) and LV were both statistically significant (p <0.0001 and p=0.0256,
respectively). Overall, supplemental FA decreased survival (p=0.0059) and LV had a significant effect on survival (p=0.0469) in a medium FA concentration-dependent manner (panels A and B of Figure 4.3.3.1.1).

As shown in panel A of Figure 4.3.3.1.1, at 1000 nM FA without LV, for each ten-fold increase in 5-FU concentration, cell survival decreased by an estimated 23.4% (p < 0.0001). The 50 nM FA group had a 10.1% higher intercept than the 1000 nM FA group (p<0.0001), whereas there was no difference in the intercepts of the other FA concentrations. If the intercepts of two lines are significantly different, then percent survival is either significantly higher or lower across all doses of 5-FU. Furthermore, without LV, cells grown in 50 nM FA had approximately 10% higher survival, across all doses of 5-FU relative to cells grown in 1000 nM FA (panel A of Figure 4.3.3.1.1). Since the interaction between FA and \( \log_{10}(5\text{-FU}) \) was not significant, the slope of the regression lines did not vary based on folate concentrations. Therefore there was no evidence that the effect of FA varied with dose of 5-FU.

As a result of the significant interaction between LV and FA, the effect of LV was found to depend on the amount of FA. Since there was a significant interaction between LV and 5-FU, LV not only changed the intercepts, but also affected the slope of the lines, meaning the effect of LV varied with dose of 5-FU (panel B of Figure 4.3.3.1.1). At 5, 500 and 1000 nM FA, LV did not have a significant effect (p=0.26, p=0.73 and p=0.18, respectively; Figure 4.3.3.1.2). However, at 50 nM FA, the addition of LV decreased cell survival by 18.0% (p<0.0001) compared to treatment in the absence of LV (Figure 4.3.3.1.2).

In conclusion, cell survival decreased with increasing concentrations of 5-FU. Percent cell survival was found to be significantly higher (across all concentrations of 5-FU) at 50 nM FA
compared to the other FA concentrations. Overall, the effect of LV was found to depend on the amount of folate. LV had no effect in the 5, 500 and 1000 nM FA groups, but decreased cell survival by 18.0% in the 50 nM FA group.

4.3.3.2 MCF-7 chemosensitivity to 5-FU

Figure. 4.3.3.2.1 Predicted percent survival for MCF-7 cells based on final regression model: percent survival = (date) (FA) (LV) (log 5-FU) (FA*LV) (log 5-FU *FA) (log 5-FU*LV), where * indicates an interaction. Panels A and B depict predicted percent survival for MCF-7 cells cultured without LV and with LV, respectively.
Figure. 4.3.3.2.2 Comparison between predicted percent survival for MCF-7 cells based on final regression model treated with 5-FU alone or 5-FU with the addition of LV among cells grown in different FA medium concentrations. Panels (A-D) depict MCF-7 cells grown in 5 nM, 50 nM, 500 nM and 1000 nM FA medium respectively, where solid and dashed lines represent percent survival with and without the addition of LV, respectively. * Indicates statistically significant difference at p<0.05.

In the MCF-7 cells, the three way interaction between FA, LV and $\log_{10}(5\text{-FU})$ was not significant (p=0.71) and was dropped from the model. However, all two-way interactions between FA and LV, between $\log_{10}(5\text{-FU})$ and FA and between $\log_{10}(5\text{-FU})$ and LV were all statistically significant (p <0.0001, p=0.0026, and p=0.0002, respectively). Due to the significant interaction between FA, 5-FU dose and LV, the effects of each variable depend on
the other variable. Overall, both FA and LV had a significant effect on cell survival (p<0.0001 and p=0.0002, respectively), was dependent on 5-FU dose and LV.

As shown in panel A of Figure 4.3.3.2.1, at 1000 nM FA without LV, for each ten-fold increase in 5-FU concentration, cell survival decreased by an estimated 27.7% (p < 0.0001). Without LV all four intercepts of the regression lines changed as FA concentrations varied. Relative to 1000 nM FA, percent survival across all doses of 5-FU was 9.9% higher at 50 nM FA (p < 0.0001), 4.9% higher at 500 nM FA (p=0.0199) and 4.9% lower at 5 nM (p=0.0171; panel A of Figure 4.3.3.2.1). In addition to changing the intercepts, the slope of the regression line (rate of decrease in survival with increasing dose of 5-FU), was found to depend on the level of FA due to the significant interaction between FA and log_{10} (5-FU). Relative to 1000 nM FA, the slope decreased for 5 nM FA (p=0.023), but the slopes of the other two FA concentrations did not differ significantly from 1000 nM Figure (panel A of 4.3.3.2.1). Furthermore, at a concentration of 5 nM of FA, each 1-unit change in log(5FU) resulted in 23.2% lower survival compared to 1000 nM FA.

Lastly, due to the significant interaction between LV and FA, the effect of LV was dependent on the amount of FA. Since there was a significant interaction between LV and 5-FU, LV not only changed the intercepts (general percent survival trend across all doses of 5-FU), but also affected the slope of the line (panel B of Figure 4.3.3.2.1). At 1000 nM FA and 5 nM FA, LV did not have a significant effect on survival (p=0.267 and p=0.642, respectively; Figure 4.3.3.2.2). However, for 50 nM and 500 nM FA, the addition of LV was found to decrease cell survival by 7.7% and 4.7% (p<0.0001 and p=0.0025, respectively) compared to treatment in the absence of LV (Figure 4.3.3.2.2).
In conclusion, in MCF-7 cells, we were able to demonstrate that cell survival decreased with increasing concentrations of 5-FU. It was also evident that the percent cell survival was found to be significantly lower (across all concentrations of 5-FU) at both the lowest (5 nM FA) and highest (1000 nM FA) concentrations, and significantly higher at 50 and 500 nM FA. LV had a significant effect on cell survival in the MCF-7 cell line, wherein the addition of LV decreased percent survival at 50 and 500 nM FA, and had no effect at 5 and 1000 nM FA.

4.3.3.3 MDA-MB-231 chemosensitivity to 5-FU

Figure. 4.3.3.3.1 Predicted percent survival for MDA-MB-231 cells based on final regression model: percent survival = (date) (FA) (LV) (log 5-FU) (FA*LV) (FA*log 5-FU), where * indicates an interaction. Panels A and B depict predicted percent survival for MDA-MB-231 cells cultured without LV and with LV, respectively.
Figure. 4.3.3.2 Comparison between predicted percent survival for MDA-MB-231 cells based on final regression model treated with 5-FU alone or 5-FU with the addition of LV among cells grown in different FA medium concentrations. Panels (A-D) depict MDA-MB-231 cells grown in 5 nM, 50 nM, 500 nM and 1000 nM FA medium respectively, where solid and dashed lines represent percent survival with and without the addition of LV, respectively. * Indicates statistically significant difference at p<0.05.

In the MDA-MB-231 cells, the three way interaction between folate, LV and log$_{10}$(5-FU) and the two-way interaction between LV and log$_{10}$(5-FU) were not significant (p=0.48 and 0.87 respectively) and were dropped from the model. However, the interaction between folate and LV and the interaction between log$_{10}$(5-FU) and folate were statistically significant (p = 0.0027 and p=0.0080, respectively). Due to the significant interaction between folate and 5-FU dose as well as between folate and LV, the effects of each variable depend on the other variable.
Importantly, overall, FA supplemental levels significantly increased survival (p<0.0001), whereas the addition of LV did not significantly affect percent survival (p = 0.76).

As demonstrated in panel A of Figure 4.3.3.3.1, at 1000 nM FA, for each ten-fold increase in 5-FU concentration, cell survival decreased by an estimated 36.2% (p < 0.0001). The intercepts of the regression lines, indicative of the general percent survival trend across all doses of 5-FU, changed as FA concentrations varied. Relative to 1000 nM FA, no significant differences in percent survival were observed for cells grown at 5 and 500 nM FA (p = 0.068 and p = 0.077, respectively; panel A of Figure 4.3.3.3.1). However, at 50 nM FA percent survival was 14.2% lower compared to 1000 nM FA across all doses of 5-FU (p < 0.0001, Figure 4.3.3.3.1(A)).

The significant interaction between FA and log_{10} (5-FU) means that in addition to having an impact on the intercept (changing percent survival), FA also affects the slope of the line for log_{10} (5-FU) vs. percent survival. This means that the relationship between dose of 5-FU and percent survival differ according to the amount of FA. Relative to 1000 nM FA, the slopes of 5 and 500 nM FA are not significantly different (p = 0.83 and p = 0.79, respectively; panel A of Figure 4.3.3.3.1). However, at 50 nM of folate, each 1-unit change in log(5FU) resulted in 27.9% lower survival compared to 1000 nM (panel A of Figure 4.3.3.3.1).

Lastly, LV did not significantly affect percent survival (p = 0.76; panel B of Figure 4.3.3.3.1). However, due to the significant interaction between LV and FA, the effect of LV was found to depend on the amount of FA. Since no interaction was found between LV and 5-FU, LV did not affect the slope of the line. At 50, 500, and 1000 nM FA LV did not have a significant effect on percent survival (p=0.728, p=0.353 and p=0.737, respectively; Figure 4.3.3.3.2).
However, at the lowest concentration of FA of 5 nM, LV was found to decrease cell survival by 5% compared to treatment in the absence of LV (p=0.0087, Figure 4.3.3.3.2).

In conclusion, as expected, cell survival decreased with increasing concentrations of 5-FU. Relative to other concentrations of folate in the medium, only the 50 nM FA concentration was found to have significantly lower survival compared to 1000 nM FA. LV did not have a significant effect on cell survival, except at the lowest concentration of 5 nM FA, where the addition of LV resulted in a significant decrease in cell survival across all levels of 5-FU.

4.3.4 The effect of FA on gene expression via quantitative RT-PCR

FA concentrations had a significant effect on TS mRNA expression in the MDA-MB-231 and MCF-7 cells but not in the HCC1937 cells (Figure 4.3.4.1). In MDA-MB-231 cells, cells cultured at 50, 500 and 1000 nM FA had significantly lower TS mRNA expression compared to cells cultured at 5 nM FA (p≤ 0.045, Figure 4.3.4.1). Similarly, in MCF-7 cells, cells cultured at 500 and 1000 nM FA had significantly lower TS mRNA expression compared to cells cultured at 50 nM FA (p= 0.0114 and p=0.0023, respectively) and cells cultured at 50, 500 and 1000 nM FA had significantly lower TS mRNA expression compared to cells cultured at 5 nM FA (p≤ 0.0099).

No significant differences in MTHFR mRNA expression were observed among cells cultured at different FA concentrations (Figure 4.3.4.2).
Figure 4.3.4.1. Fold change in TS expression on day 5 relative to day 0 in MDA-MB-231, MCF-7 and HCC1937 cell lines cultured at 5, 50, 500 and 1000 nM FA medium. Medium FA was only found to have a significant effect on TS expression in the MDA-MB-231 and MCF-7 cell lines and not in the HCC1937 cell line. Means statistically significant between different medium FA groups within the same cell line are denoted with different letters at p<0.05. Results expressed as mean ± SEM.

Figure 4.3.4.2. Fold change in MTHFR expression on day 5 relative to day 0 in MDA-MB-231, MCF-7 and HCC1937 cell lines cultured at 5, 50, 500 and 1000 nM FA medium. Medium FA did not have a significant effect on MTHFR expression in all 3 cell lines. Results expressed as mean ± SEM.
Medium FA concentrations had a significant effect on DHFR mRNA expression in MCF-7 cells but not in MDA-MB-231 and HCC1937 cells (Figure 4.3.4.3). MCF-7 cells cultured at 50, 500 and 1000 nM FA had significantly lower DHFR mRNA expression compared to cells cultured at 5 nM FA (p= 0.0375, p=0.0100 and p=0.0018, respectively; Figure 4.3.4.3).

Figure 4.3.4.3. Fold change in DHFR expression on day 5 relative to day 0 in MDA-MB-231, MCF-7 and HCC1937 cell lines cultured at 5, 50, 500 and 1000 nM FA medium. Medium FA was only found to have a significant effect on DHFR expression in the MCF-7 cell line but not in the MDA-MB-231 and HCC1937 cell lines. Means statistically significant between different medium FA groups within the same cell line are denoted with different letters at p<0.05. Results expressed as mean ± SEM.

Medium FA concentrations had a significant effect on MRP1 mRNA expression in MCF-7 cells but not in MDA-MB-231 and HCC1937 cells (Panel A of Figure 4.3.4.4). In MCF-7 cells, cells cultured at 500 nM FA had significantly lower MRP1 mRNA expression compared to cells cultured at 5 nM FA (p= 0.0249, Panel A of Figure 4.3.4.4); however, no significant differences in mRNA expression were observed among cells cultured at other FA concentrations. No
significant differences in \textit{MRP5} mRNA expression were observed among cells cultured at different FA concentrations (Panel B of Figure 4.3.4.4).

![Graph showing fold change in MRP1 and MRP5 expression on day 5 relative to day 0](image)

**Figure 4.3.4.4.** Fold change in MRP1 (panel A) and MRP5 (panel B) expression on day 5 relative to day 0 in MDA-MB-231, MCF-7 and HCC1937 cell lines cultured at 5, 50, 500 and 1000 nM FA medium. Medium FA concentrations had a significant effect on \textit{MRP1} expression in the MCF-7 cell line but not in the MDA-MB-231 and HCC1937 cell lines. Medium FA concentrations did not have a significant effect on \textit{MRP5} mRNA expression in all 3 cell lines. Means statistically significant between different medium FA groups within the same cell line are denoted with different letters at $p<0.05$. Results expressed as mean ± SEM.

Medium FA concentrations had a significant effect on \textit{MRP8} mRNA expression in MCF-7 cells; however, \textit{MRP8} mRNA was not detected in MDA-MB-231 and HCC1937 cells (Figure 4.3.4.5). Similarly to \textit{MRP1} expression, MCF-7 cells cultured at 500 nM FA had significantly lower \textit{MRP8} mRNA expression compared to cells cultured at 5 nM FA ($p=0.0298$, Figure 4.3.4.5) but no significant differences in expression were observed among cells cultured at other FA concentrations.
Figure 4.3.4.5. Fold change in MRP8 expression on day 5 relative to day 0 in MCF-7 cells cultured at 5, 50, 500 and 1000 nM FA medium. MRP8 mRNA expression was not detected in MDA-MB-231 and HCC1937 cell lines. Medium FA concentrations had a significant effect on MRP8 expression in the MCF-7 cell line. Statistically significant differences among means of medium FA are denoted with different letters at \( p < 0.05 \). Results expressed as mean ± SEM.

4.3.5 Summary

As summarized in Table 4.3.5, supplemental FA levels resulted in higher growth rates in all three cell lines compared to the 5 nM FA deficiency level. Intracellular folate concentrations were significantly different among the four FA treatment levels at each time point.

Supplemental FA affected chemosensitivity to 5-FU in a cell-specific manner, whereby it increased chemosensitivity in the HCC1937 cell line, decreased chemosensitivity in the MDA-MB-231 cell line and followed an inverted U shape in the MCF-7 cell line. The addition of LV decreased survival in a FA medium concentration dependent manner (Table 4.3.5). FA supplementation did not significantly change mRNA expression of the interrogated genes in the HCC1937 cell line. In the MCF-7 cell line, however, supplemental FA resulted in lower TS,
DHFR, MRP1 and MRP8 mRNA expression. In the MDA-MB-231 cell line, supplemental FA only decreased TS mRNA expression (Table 4.3.5).

Table 4.3.5. Summary of growth curve, intracellular folate concentration, chemosensitivity and relative gene expression.

<table>
<thead>
<tr>
<th>Assay</th>
<th>HCC1937</th>
<th>MCF7</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth curve</td>
<td>Cell growth at 500 nM FA = 1000 nM FA &gt; 50 nM FA &gt; 5 nM FA</td>
<td>Cell growth at 50 nM FA = 500 nM FA = 1000 nM FA</td>
<td>Cell growth at 1000 nM FA &gt; 500 nM FA = 50 nM FA &gt; 5 nM FA</td>
</tr>
<tr>
<td>Intracellular Folate</td>
<td>Folate concentrations were significantly different among cells cultured in 4 FA levels.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemosensitivity</td>
<td>Overall, supplemental FA <strong>decreased</strong> survival and at 50 nM FA, the addition of LV decreased cell survival by 18.0%</td>
<td>Cell survival was lowest at 5 and 1000 nM FA, and significantly higher at 50 and 500 nM FA. The addition of LV decreased percent survival at 50 and 500 nM FA.</td>
<td>Overall, supplemental FA <strong>increased</strong> survival. LV decreased cell survival by 5% at 5 nM FA.</td>
</tr>
<tr>
<td>Gene expression via quantitative RT-PCR</td>
<td>No significant changes.</td>
<td>TS, DHFR, MRP1 and MRP8 mRNA expression decreased with increasing FA.</td>
<td>TS mRNA expression was significantly lower at 50, 500 and 1000 nM FA compared to 5 nM FA.</td>
</tr>
</tbody>
</table>

4.4 Discussion

4.4.1 Growth curves

High intakes of folate and FA from dietary sources and prevalent supplement use in breast cancer patients are concerning, given emerging evidence on the role of folate in breast cancer. Recent epidemiologic studies have reported an increased risk of breast cancer with high folate levels [23-28] and two recent animal studies have suggested that FA supplementation increases breast cancer risk [29, 126]. These results are troubling because FA
supplementation can promote the growth of (pre)neoplastic lesions in breast tissue by providing an increased amount of nucleotide substrates for DNA synthesis, leading to accelerated tumor growth and by *de novo* methylation of promoter CpG islands of tumor suppressor genes leading to consequent gene silencing [150].

As such, we assessed the effect of FA supplementation on the proliferation of the MCF-7, MDA-MB-231 and HCC1937 cell lines. The four folate concentrations that were assessed were 5 nM FA (chosen as a deficiency level), 50 nM FA (chosen to parallel mean serum levels in the North American population post-fortification [20]), 500 and 1000 nM FA (10x and 20x control level, respectively). Cells at higher FA concentrations were expected to exhibit higher cellular proliferation than cells cultured at lower FA concentrations, primarily due to increased supplies of nucleotides needed for DNA synthesis.

To our knowledge, there are no other studies that have assessed cellular proliferation in response to FA supplementation in breast cancer cell lines. In all 3 cell lines, MCF-7, MDA-MB-231 and HCC1937, cells cultured at 5 nM FA (deficiency model) exhibited slower growth rates compared with those cultured at 50, 500 and 1000 nM FA (Figure 4.3.1). FA supplementation at 1000 nM was associated with higher growth rates than 50 nM in the HCC1937 and MDA-MB-231 cell lines but not in the MCF-7 cell line. No difference in growth rates was observed between 500 nM and 1000 nM in the HCC1937 and MCF-7 cell lines but in the MDA-MB-231 cell line, FA supplementation at 1000 nM was associated with a higher growth rate than 500 nM.

Our failure to observe a dose-responsive relationship may be explained by a threshold effect, whereby once a certain concentration of folate is present in the medium, either
enzymes involved in folate uptake, retention or nucleotide biosynthesis are saturated or
downregulated and additional folate in the medium would not result in increased proliferation
[9]. At lower doses of medium FA, uptake is mediated by PCFT and RFC, however at higher
doses of FA such as 500 and 1000 nM, transporters become saturated and FA can enter the cell
through passive diffusion, which is much slower [12]. In addition to saturation, these carriers
responsible for FA uptake may also become downregulated. Indeed, Ashokkumar and
colleagues showed that carrier-mediated uptake in cells maintained in medium
oversupplemented with FA was significantly lower compared to cells maintained in folate-
sufficient medium, mediated in part by transcriptional regulation modified by FA [141].
Intracellularly, at high FA levels, there is increased competition for FPGS resulting in FPGS
saturation and the efflux of FA [9]. This is consistent with human data, where no significant
differences in FA concentration were observed in subjects consuming 5 mg FA compared to 1
mg FA [198, 199].

This ‘threshold effect’ may explain why by day fifteen there is no difference in
proliferation between the upper three concentrations of folate (50, 500 and 1000 nM FA) in
the MCF-7 cell lines and no difference in proliferation between 500 and 1000 nM FA in the HCC
1937 cell line. Cells cultured at 5 nM FA exhibited approximately a four-fold reduction in
proliferation in the HCC1937 and MCF-7 cell lines and approximately a ten-fold reduction in
proliferation in the MDA-MB-231 cell line. Although a statistically significant difference was
detected in cells cultured at 50 nM FA compared to upper FA concentration in the HCC1937
and MDA-MB-231 cell lines, the small difference may not be physiologically relevant.
Furthermore, results from the proliferation assay indicate that a threshold effect of FA may result in the observed proliferation rates, and 50 nM may be the physiological threshold level.

Our gene expression data fits the growth curve observations in part for the MCF-7 cells. No significant differences were observed in DHFR mRNA expression of MCF-7 cells cultured at 50, 500 and 1000 nM FA. Since DHFR is needed for the conversion of FA to DHF and THF, these data suggest that no difference in proliferation among these three concentrations of FA in MCF-7 cells should be observed, which fits our observation. On the other hand, TS mRNA expression is significantly higher in MCF-7 cells cultured at 50 nM FA compared to 500 and 1000 nM FA. Since FA supplementation was found to decrease TS expression, this may be explained by a higher proportion of cells cultured at higher FA concentrations being in plateau phase compared to log phase at lower FA concentrations. If this was the case, since the TS mRNA expression level at 50 nM FA was significantly different from the other concentrations of FA, then one would also expect to see a difference in proliferation at 50 nM FA compared to other FA concentrations. However, the growth curve data for MCF-7 cells do not fit this model.

Similarly, in the MDA-MB-231 cell line, higher proliferation was observed at 1000 nM FA compared to 50 and 500 nM FA. However, no differences in TS mRNA expression were observed among cells cultured at 50, 500 and 1000 nM FA. Additionally, no differences in the expression of interrogated genes were observed of HCC1937 cells. Furthermore, perhaps other genes or their synergistic effect may be responsible for our growth curve observations.
4.4.2 Intracellular folate concentrations varied based on FA concentrations in the medium

It was important to ascertain whether FA supplementation in the media increased intracellular folate concentrations because changes in intracellular FA levels may affect the expression of enzymes involved in folate metabolism, methylation reactions, thereby modulating chemosensitivity to 5-FU. Indeed, we can conclude that by day five, intracellular concentrations were significantly different among the four folate concentrations, with the exception of the 5 nM and 50 nM groups in the MCF-7 cell line. Consequently, changes seen in percent survival may be attributed to intracellular folate concentrations.

Intracellular folate concentrations were significantly different among cells cultured in 4 different FA levels in all 3 cell lines, where cells cultured in lower FA medium had significantly lower intracellular folate concentrations than the corresponding cells cultured in medium containing higher levels of FA (Figure 4.3.2). Since intracellular folate levels vary based on medium FA concentrations, proliferation results are likely to be the result of changes in enzyme expression levels involved in nucleotide biosynthesis, and not folate uptake. Interestingly, FA concentrations had a significant effect on TS mRNA expression, whereby cells cultured at higher FA concentrations had significantly lower TS mRNA expression compared to cells cultured at deficiency level (Figure 4.3.4.1). Since TS expression is significantly related to the cell doubling time [200], meaning higher TS expression results in increased proliferation, our results are puzzling. An explanation for the lower TS mRNA expression we observed at higher FA concentrations is that the expression of TS varies inversely when cell-growth progresses from late-log to plateau phase (NCBI, Gene ID: 7298). Since cells cultured at higher
FA concentrations proliferated more rapidly compared to 5 nM, more cells were in plateau phase at higher FA concentrations, resulting in lower TS mRNA expression we observed.

In MCF-7 cells we also observed significantly lower DHFR mRNA expression for cells cultured at 50, 500 and 1000 nM FA compared to 5 nM FA. Due to the function of DHFR, one would expect to see lower intracellular folate stores with lower DHFR expression. However, the intracellular folate data suggest that folate concentrations are higher at supplemental FA despite the lower DHFR expression. A mechanism that may explain these observations may be the negative feedback inhibition of DHFR by DHF. MCF-7 cells cultured at 50, 500 and 1000 nM FA may have significantly higher DHF levels that would result in the downregulation of DHFR. Upon the inhibition of DHFR, cells cultured at 500 and 1000 nM FA may still contain higher intracellular folate stores because DHFR is post-transcriptionally regulated and a pool of mRNA transcripts may still be translated. Since there is increased FA substrate availability at 500 and 1000 nM FA, this would result in the higher intracellular folate stores we observed compared to cells cultured at 5 and 50 nM FA.

Interestingly, cells cultured at the lowest FA concentration of 5 nM still demonstrated measurable levels of intracellular folate that dropped to 5-10% of the day zero value in all three cell lines by day 15. Although some studies have reported intracellular folate levels upon treatment with LV and antifolate based chemotherapeutics [201], there are limited published in vitro studies that have reported intracellular folate levels due to FA supplementation in cancer cells. Using MDA-MB-435 cell line, Cho and colleagues have reported intracellular folate concentrations ranging from 6 to 16 ng folate per 5 million cells cultured in 20 nM and 2.3 µM FA medium, respectively [176]. Subsequently, another study examining GGH inhibition in
HCT116 and MDA-MB-435 cell lines reported much lower intracellular folate concentrations ranging from 0.6 to 1.5 ng folate per 5 million cells [177]. There are no published results on intracellular folate stores in MCF-7, MDA-MB-231 and HCC1937 cells grown in FA supplemented medium. Since our values are reported in ng folate per 10 million cells, the range of intracellular folate stores observed in the HCC1937 and MDA-MB-231 cell lines fall within the ranges reported by the aforementioned studies. MCF-7 intracellular folate stores are surprisingly higher compared to the other two cell lines.

A plausible explanation for the differences in intracellular folate concentrations among the three cell lines cultured at the same medium FA level may be due to metabolic differences, namely rates of proliferation among the cell lines. From the growth curve data, we can conclude that MCF-7 cells had the fastest growth rates, followed by MDA-MB-231 cells and HCC1937 cells. Since MCF-7 cells had significantly higher intracellular folate concentrations compared to the other two cell lines, we can speculate that the high folate stores in MCF-7 cells were adequate to meet its metabolic demand. However, HCC1937 cells had significantly higher intracellular folate stores compared to MDA-MB-231 cells, but exhibited lower growth rates compared to MDA-MB-231 cells. This observation may be attributed to differences in doubling time of HCC1937 and MDA-MB-231 cells. HCC1937 cells have a doubling time of approximately 60 hours, whereas MDA-MB-231 cells have a doubling time of 24 hours. Despite the high intracellular folate levels in the HCC1937 cells, these cells have lower metabolic needs due to their longer doubling time compared to MDA-MB-231 cells. On the other hand, low intracellular folate pools meet the high metabolic demand of MDA-MB-231 cells, resulting in more than 2x total cell number compared to the HCC1937 cells.
The differences in the ranges of intracellular folate can also be explained by the differential expression of enzymes responsible for folate uptake, RFC, PCFT and folate receptor alpha (FRα) among the three cell lines. All three of these receptors are ubiquitously expressed in tissues and tumors, however in peripheral tissues including the breast, their expression is reduced [202]. In normal breast tissue, RFC expression is higher than PCFT and FRα [203]. However, in cancer cells, the expression of these receptors becomes considerably altered. For instance, RFC expression in MCF-7 cells has been shown to be significantly upregulated compared to RFC expression in MDA-MB-231 cells [204]. Similarly, various groups have also shown that RFC is detectable in MCF-7 cells and undetectable in MDA-MB-231 at the mRNA level [205-207]. In contrast, TNBC cell lines MDA-231 and HCC1937 have higher expression of FRα compared to ER+ cell lines like MCF-7 [208]. Within TNBC, HCC1937 has a four-fold higher FRα expression compared to MDA-MB-231 [208].

Since RFC is the major folate transporter in mammals and transports folates from blood into cells of the peripheral tissues [209], higher RFC expression would cause significantly higher intracellular stores. Our observations are in accord with this assumption, as our MCF-7 intracellular stores ranged from 10-90 ng folate per 10 million cells compared to a range of 2-13 and 0.5-3.7 ng folate per 10 million cells in the HCC1937 and MDA-MB-231 cell lines respectively. Even though HCC1937 and MDA-MB-231 cells had higher expression of FRα, lower folate uptake was observed due to the upregulation of RFC expression in MCF-7 cells. The higher intracellular folate stores in HCC1937 cells compared to MDA-MB-231 cells may be explained by the previously reported four-fold higher FRα expression compared to MDA-MB-231 cells.
4.4.3 *In vitro* chemosensitivity to 5-FU and gene expression via quantitative RT-PCR

4.4.3.1 HCC1937 cells

Overall, supplemental FA decreased survival (p=0.0059) and LV had a significant effect on survival (p=0.0469) in a folate dependent manner. Additionally, the interaction between FA and LV and the interaction between \( \log_{10} (5\text{-FU}) \) and LV were statistically significant (p <0.0001 and p=0.0256, respectively), whereas the interaction between \( \log_{10} (5\text{-FU}) \) and FA was not significant (p=0.34). The interactions between FA and LV and between \( \log_{10} (5\text{-FU}) \) and LV are of biological significance, as both LV and FA contribute to intracellular 5, 10-methyleneTHF stores, which is a substrate for the inhibitory ternary TS-5FdUMP-5, 10-methyleneTHF complex. Due to the significant interaction between LV and FA, the effect of LV was found to depend on the amount of folate. The addition of LV only had a significant effect at the 50 nM FA whereby cell survival was decreased by 18.04% compared to treatment in the absence of LV.

HCC1937 cells have low RFC and PCFT expression, and a significantly higher expression of FRα compared to MCF-7 and MDA-MB-231 cells [208]. FRα exhibits high affinity for all forms of folate; however, their affinity is the highest for FA [9]. Since folate uptake is predominantly carried out by FRα in HCC1937 cells, at higher concentrations of 500 and 1000 nM FA, these receptors may be saturated with FA and consequently LV may not be taken up into the cell and would not affect survival as we observed. However, at lower FA concentrations of 5 and 50 nM, some FRα may be available for LV uptake and lower survival was expected. Indeed, at 50 nM FA the addition of LV decreased cell survival by 18.04%, but we did not observe a
significant change in survival at 5 nM. This may be due to Type II error since our sample size for 5 nM FA was significantly smaller due to technical limitations of the assay combined with the poor proliferation of HCC1937 cells in 5 nM FA. Furthermore, we may not have had enough power to detect the change in survival as we did at 50 nM FA.

Without LV, percent cell survival was found to be significantly higher at 50 nM FA compared to 1000 nM FA. The results obtained from treatment with 5-FU alone were unexpected. Since no difference in the expression of TS, DHFR, MTHFR, MRP1, 5 and 8 were observed through qRT-PCR analysis, these observations may be explained by the BRCA1 status of HCC1937 cells. Similarly to MDA-MB-231 cells, HCC1937 cells are TNBC, with the major distinction of having a homozygous BRCA1 5382C mutation [190]. BRCA1 in combination with other tumor suppressor proteins forms a large multi-subunit protein complex known as the BRCA1 associated genome surveillance complex (BASC), with the primary function of repairing double stranded DNA breaks and base pair mismatches by coordinating the actions of damage-sensing proteins and executive repair proteins [210, 211]. Furthermore, the mutation of BRCA1 in the HCC1937 cells, likely impairs the repair of damaged DNA.

In a folate rich environment, there would be adequate 5,10-methylene THF stores to form the inhibitory ternary complex resulting in depleted nucleotide stores. Consequently, the ratio of 5FdUTP (metabolite of 5-FU that may be incorporated into DNA) to ddUTP would become higher, resulting in increased misincorporation of 5FdUTP. Since these cells have impaired DNA repair due to the mutation in BRCA1, these mutations may likely accrue until apoptosis becomes activated resulting in cell death. Therefore, at higher FA concentrations in this cell line, the main cytotoxic mechanism of 5-FU may be the incorporation of 5FdUTP into
DNA, which due to its compromised DNA repair pathway may activate apoptosis resulting in the lower survival we observed.

However, survival was also significantly lower at 5 nM FA compared to 50 nM FA. Due to the low intracellular folate levels at 5 nM, there may be low stores of 5,10-methyleneTHF that may not be able to meet the suppressive demand of TS, resulting in increased unbound 5FdUMP stores. 5FdUMP can be converted to 5FdUTP, which is the cytotoxic metabolite that can be incorporated into DNA. Furthermore, at 5 nM FA, inadequate intracellular folate stores may result in increased 5FdUMP stores that can be converted into 5FdUTP, thereby shunting the cytotoxicity of 5-FU towards DNA misincorporation. Similarly, to the situation that may arise in a high folate environment, cells grown at 5 nM may not have been able to repair the DNA damage caused by the incorporation of 5FdUTP resulting in lower survival through the activation of apoptosis. At 50 nM FA, intracellular folate levels were likely optimal to reduce the efficacy of the aforementioned cytotoxic pathways, whereby intracellular folate levels were optimal to produce a lower ratio of 5FdUTP to ddUTP, resulting in decreased repair demand and higher survival which was in accordance with our observations.

4.4.3.2 MCF-7 cells

In the MCF-7 cell line, both FA and LV had a significant effect on survival (p<0.0001 and p=0.0002, respectively). Additionally, all two-way interactions between FA and LV, log_{10} (5-FU) and between FA and log_{10} (5-FU) and LV were statistically significant (p <0.0001, p=0.0026, and p=0.0002, respectively). The interaction among FA, LV and log_{10} (5-FU) is of biological significance, as both FA and LV contribute to intracellular 5, 10-methyleneTHF stores, which is needed for the inhibitory ternary TS-5FdUMP-5, 10-methyleneTHF complex. Due to the
significant interaction between LV and FA, the effect of LV was found to depend on the amount of FA. Unlike the MDA-MB-231 cell line, at 5 and 1000 nM FA, LV did not have a significant effect, but at 50 nM and 500 nM FA, the addition of LV was found to decrease cell survival by 7.7% and 4.7% compared to treatment in the absence of LV, respectively.

As suggested earlier, this may be due to modulation of LV uptake by FA and folate transporter expression. RFC expression in MCF-7 cells has been shown to be significantly upregulated compared to the other two TNBC cell lines [204]. Since RFC has a markedly higher affinity for reduced folates relative to FA, it would be the main transporter of LV into the cell across all concentrations of FA [9]. At 1000 nM FA, RFCs may be saturated and additional LV may not be needed to increase intracellular 5, 10-methyleneTHF stores. Indeed, intracellular folate data suggests that there is no difference in intracellular folate concentration between 1000 nM on day 5 and baseline (grown in 2.3 µM FA), suggesting 1000 nM is adequate to maximize intracellular folate stores. On the other hand, the addition of 5 µM LV to cells grown in 5 nM FA, would likely cause a spike in LV uptake through the unsaturated RFCs. But why would this not cause a decrease in survival? Perhaps due to rapidly increased efflux of LV. Analysis of MRP1 mRNA expression through qRT-PCR revealed that MRP1 expression was significantly higher in cells cultured at 5 nM FA relative to the other three concentrations. MRP1 can regulate intracellular folate stores by exporting monoglutamate or short-chain polyglutamate forms of folate such as LV, resulting in a decrease in the efficacy of LV to potentiate the cytotoxicity of 5-FU [99]. Therefore, increased efflux of LV may be an explanation for why LV did not affect survival at 5 nM. Finally, LV was observed to decrease survival at 50 and 500 nM FA. This may be explained by the availability of unbound RFCs,
leading to LV uptake, increased 5, 10-methyleneTHF stores and increased cytotoxicity. Additionally, MRP1 expression at 50 and 500 nM FA was found to be significantly lower than at 5 nM FA, resulting in lower efflux of LV and higher sensitivity to 5-FU which are in accord with our observations.

Without LV, percent cell survival was found to be significantly lower across all doses of 5-FU at both the lowest (5 nM FA) and highest (1000 nM FA) concentrations compared to 50 and 500 nM FA. This may be due in part to differential mRNA expression of interrogated genes. Significant changes in TS, DHFR, MRP1 and MRP8 gene expression were observed in the MCF-7 cell line: 1) TS expression was the highest in the 5 nM group, significantly lower in the 50 nM group and lowest in the 500 and 1000 nM groups; 2) DHFR expression was significantly higher at 5 nM FA compared to the other three FA concentrations; 3) MRP1 expression was significantly higher at 5 nM compared to 500 nM, and no difference in MRP1 expression was observed among 50, 500 and 1000 nM FA groups; and 4) MRP8 expression was significantly higher at 5 nM FA compared to 500 nM FA, whereas no significant difference was detected at 50 and 1000 nM FA relative to 5 and 500 nM.

Since higher TS expression has been associated with decreased sensitivity to 5-FU [182], the 5 nM group should have the highest survival since it had the highest TS expression. However, cells grown at 5 nM also had a significantly higher DHFR expression compared to the other three FA concentrations. DHFR is needed to convert FA into DHF, then again to convert DHF to THF, which can be converted to 5, 10-methyleneTHF resulting in increased cytotoxicity through the formation of the inhibitory ternary complex. Furthermore, higher DHFR levels would increase sensitivity to 5-FU. Finally, at 5 nM FA, MRP1 and MRP8 expression were
significantly higher than at 500 nM. High MRP1 expression would result in increased efflux of monoglutamate or short-chain polyglutamate forms of folate such as LV [99], whereas high MRP8 expression would result in increased efflux of 5-FU, 5-fluoro-2′-deoxyuridine, and 5-fluoro-5′-deoxyuridine alike [98].

Regardless of high TS, MRP1 and MRP8 expression, cells grown in 5 nM FA had the lowest survival (highest chemosensitivity). It appears that DHFR levels had the most significant effect on survival in MCF-7 cells. This is supported by survival at 50 nM FA, where TS expression was significantly higher than the upper two FA concentrations, no significant change in MRP1 and MRP8 expression was observed compared to the other three FA concentrations, but DHFR expression was significantly lower relative to 5 nM FA. Therefore, the only difference between 5 nM and 50 nM FA was the downregulation of DHFR expression at 50 nM, which might have compromised 5, 10-methyleneTHF stores and decreased sensitivity to 5-FU. Indeed, survival was the highest at 50 nM FA. This suggests that FA levels over 50 nM FA result in a downregulation of DHFR, leading to increased survival at 50, 500 and 1000 nM FA compared to 5 nM. Analysis of gene expression for 500 and 1000 nM FA did not yield any significant differences between the two. A plausible mechanism for why cells at 1000 nM had a 4.9% decrease in survival compared to 500 nM FA may be simply the difference in intracellular folate. By day 5, intracellular folate stores at 1000 nM are significantly higher compared to 500 nM. Consequently, at 1000 nM FA, there would be more substrate for DHFR to act on, resulting in increased 5,10-methyleneTHF stores and increased sensitivity to 5-FU compared to 500 nM.
4.4.3.3 MDA-MB-231 cells

In the MDA-MB-231 cell line, overall, FA supplemental levels significantly increased survival ($p<0.001$), whereas the addition of LV did not significantly affect percent survival ($p = 0.76$). Additionally, the interaction between FA and LV and the interaction between $\log_{10}$ (5-FU) and FA were statistically significant ($p = 0.0027$ and $p=0.0080$, respectively). The interaction among FA, LV and $\log_{10}$ (5-FU) is expected, as both FA and LV are precursors to 5, 10-methyleneTHF, critical for the formation of TS-5FdUMP-5, 10-methyleneTHF inhibitory complex. However, the observation that LV did not significantly affect percent survival was unexpected, since LV is a precursor for 5, 10-methyleneTHF and should potentiate the cytotoxicity of 5-FU. An explanation for this observation may be impaired uptake of LV. As mentioned previously, MDA-MB-231 cells have low PCFT and FRα expression compared to the other two cell lines, and undetectable RFC at the mRNA level [205-207]. Since RFC is the main transporter, with a markedly higher affinity for reduced folates relative to FA, it would be the main transporter of LV into the cell [9]. Due to the extremely low expression of RFC, LV was likely not taken up into the cell and consequently did not affect cell survival at 50, 500 and 1000 nM folate. However, at the lowest concentration of FA at 5 nM, LV was found to decrease cell survival by 5% compared to treatment in the absence of LV ($p=0.0087$). At 5 nM FA (lowest), PCFT, RFC, but most importantly FRα receptors may not have been saturated with FA and LV was able to bind FRα and enter the cell. Even though FRα exhibits the highest affinity for FA, due to low FA concentration (5 nM) and high LV concentration (5 µM), LV was free to bind the receptors.
Relative to other concentrations of folate in the medium, only the 50 nM FA concentration was found to have significantly lower survival compared to 1000 nM FA. This demands the question why cells at 5 nM also did not have lower survival. Different FA levels in the medium may have changed enzymes critical for the cytotoxicity of 5-FU or induced MRPs. Indeed, at 5nM FA, qRT-PCR analysis revealed TS expression was significantly higher than at the other three concentrations of FA. As discussed earlier, higher TS expression has been associated with decreased sensitivity to 5-FU, because there would not be sufficient 5FdUMP to bind the increased TS stores, resulting in proportionally more TS to supply substrates required for DNA synthesis [182]. No significant changes were observed in MTHFR, DHFR, MRP1 and MRP5 expression levels in the MDA-MB-231 cells. Additionally, mRNA levels for MRP8 were undetectable for this cell line. Furthermore, higher survival at 5 nM relative to 50 nM may be attributed to increased TS expression. Although no significant difference in TS expression was detected among 50, 500 and 1000 nM FA, this may be due to Type II error resulting from a small effect size and plate to plate variation. In a high folate environment, unbound TS has been shown to dissociate from its promoter, resulting in increased transcription and increased TS stores [178]. Furthermore, the higher survival at 500 and 1000 nM relative to 50 nM may be attributed to increased TS expression which we have failed to capture due to Type II error.
5 Chapter 5: General Discussion, Strengths & Limitations, Future Directions and Conclusions

5.1 Summary and general discussion

Considering the rising incidence of breast cancer in women worldwide, high FA containing supplement use by breast cancer patients and the prevalence of 5-FU in chemotherapy formulations used in breast cancer patients, it is important to investigate whether FA supplementation may reduce chemosensitivity of breast cancer cells to 5-FU. Although in vitro and animal studies have demonstrated folate deficiency to increase chemosensitivity of cancer cells to 5-FU, the effect of FA supplementation has not been established. There are no studies, to the best of our knowledge, that have evaluated whether FA supplementation influences chemosensitivity of breast cancer cells to 5-FU in an in vitro model. Elucidating optimal FA levels in breast cancer patients during treatment with 5-FU-based chemotherapy would be beneficial in maximizing treatment efficacy, minimizing toxicities, thereby reducing the potential for negative side effects and overall cost burden on the health care system.

As such, an in vitro study was conducted using three breast cancer cell lines MCF-7, MDA-MB-231 and HCC1937, which reflect three different molecular subtypes of breast cancer to investigate whether FA supplementation modulates chemosensitivity of human breast cancer cells to 5-FU in an in vitro model and to elucidate potential mechanisms responsible for this effect. The four folate concentrations that were assessed were 5 nM FA (chosen as a
deficiency model), 50 nM FA (control-chosen to parallel mean serum levels in the North American population [20]), 500 and 1000 nM FA (10x and 20x control levels, respectively). The hypothesis of this study was that FA supplementation would decrease 5-FU chemosensitivity of human breast cancer cells in vitro.

We are the first to assess the cellular proliferation due to FA supplementation in the MCF-7, MDA-MB-231 and HCC1937 cell lines. In all 3 cell lines, cells cultured at 5 nM FA exhibited a slower growth rate compared with those cultured at 50, 500 and 1000 nM FA. Generally, FA supplementation was associated with higher growth rates in the three cell lines in a cell-specific manner and a dose-responsive relationship was not observed. Our failure in observing a dose-responsive relationship with supplemental FA is consistent with previous in vitro [141] and human studies [198, 199], and may be explained by the saturation or downregulation of enzymes responsible for FA uptake and retention at high FA levels.

Further intracellular folate measurements on day zero, five, ten and fifteen confirmed that intracellular folate concentrations were significantly different among cells cultured in 4 different FA levels in all 3 cell lines, where cells cultured in lower FA medium had significantly lower intracellular folate concentrations than the corresponding cells cultured in medium containing higher levels of FA. Since intracellular folate levels vary based on medium FA concentration, proliferation results are likely to be the result of enzyme expression levels in nucleotide biosynthesis, and not folate uptake.

FA was found to have a significant effect on sensitivity to 5-FU in all three cell lines in a cell specific manner. Similarly, LV, administered clinically to potentiate the cytotoxicity of 5-FU, was found to significantly decrease survival in all three cell lines in a folate-dependent and cell
specific manner. In MDA-MMB-231 cells LV decreased survival by 5% at 5 nM FA, whereas in MCF-7 cells LV only decreased survival at 50 and 500 nM FA by 7.7% and 4.7%, respectively, and in HCC1937 cells LV decreased survival by 18% at 50 nM FA. The discrepancies in sensitivity to 5-FU in the presence of LV among the cell lines may be explained by the differential uptake of LV into these cells due to differential expression of RFC, PCFT and FRα among the three cell lines and the induction of MRPs as a result of varying intracellular folate concentrations.

Without LV, FA supplementation influenced chemosensitivity of human breast cancer cells to 5-FU in a cell-specific manner. FA supplementation significantly decreased 5-FU chemosensitivity in MDA-MB 231 cells, significantly increased 5-FU chemosensitivity in HCC1937 cells and followed an inverted U shape in MCF-7 cells, where cell survival was found to be significantly lower (across all concentrations of 5-FU) at both the lowest (5 nM FA) and highest (1000 nM FA) concentrations, and significantly higher at 50 and 500 nM FA. Semi-quantitative real time RT-PCR demonstrated significantly lower TS and DHFR expression with increasing FA concentrations in MDA-MB-231 and MCF-7 cell lines, and significant changes in MRP1 and MRP 8 expression in MCF-7 cells. The differential sensitivity of these cell lines to 5-FU may be attributed to the complex interplay between differential FA and 5-FU uptake, the modulation of TS and DHFR expression by FA, and the induction of MRPs.

Our findings highlight the necessity for additional studies examining the effect of FA supplementation on chemosensitivity to 5-FU in human breast cancer cells. A reduction in sensitivity to 5-FU observed in MDA-MB-231 cells and the inverted U shaped curve of survival in MCF-7 cells due to high FA levels is of particular concern in the context of breast cancer patients as 67-87% report supplement use after diagnosis [22]. Our data demonstrating high
proliferation rates at 50 nM FA or higher is also of concern, given the purported tumorigenic effect of FA supplementation on breast cancer. Our study therefore highlights the need for improved health care guidelines regarding FA intake, particularly among the highly susceptible population of breast cancer patients in whom optimal FA levels are critical to maximize the efficacy of 5-FU based treatments that are the cornerstone of chemotherapy formulations used in the treatment of breast cancer.

5.2 Strengths and limitations

As discussed in sections 2.1.2 and 2.1.3, breast cancer carcinogenesis is a very complex process that results in a very heterogeneous phenotype. Therefore, currently the best approach to model breast cancer is to use an integrated approach. A main strength of this study is the use of 3 human breast cancer cell lines, representing 3 different molecular subtypes of breast cancer, which allowed us to capture the heterogeneity in treatment response based on subtype. Breast cancer cell lines have been the most widely used models to elucidate genes and signaling pathways that regulate breast cancer progression [212]. Other benefits to using human cell lines include reproducible results under well-defined experimental conditions and increased relevancy to human breast cancer than using rodent models [212]. A study by Gray et al [213] demonstrated that a panel of 51 breast cancer cell lines (including HCC1937, MCF-7 and MDA-MB-231) share many of the recurrent genomic abnormalities observed in primary tumors.

Since patients presenting with more aggressive subtypes of breast cancer, such as TNBC and metastatic disease are the most reliant on chemotherapy treatment, another benefit of using the cell lines in our study is that both MCF-7 and MDA-MB-231 were derived from
metastatic sites (pleural effusion) and are likely representative of more aggressive disease. However, despite these strengths, a major limitation of in vitro work is that it does not capture the breast microenvironment and the interaction with stromal cells that is critical for breast cancer progression.

Our study assessed the cellular growth, intracellular folate stores and chemosensitivity at 4 different FA concentrations that were chosen to model deficiency, mean human serum FA levels as control, 10x and 20x control to approximate supplemental levels. This allowed for us to test whether a dose-responsive relationship was present and to make it more generalizable to humans. However, despite our efforts to approximate physiological levels of FA, 5-FU, and LV; our model still creates an artificial environment where experimental agent levels will differ from in vivo conditions. Additionally, the assays used in intracellular folate determination and chemosensitivity were all extensively used in previous in vitro 5-FU chemosensitivity experiments [175-177].

The use of validated assays for RNA extraction, cDNA synthesis and semi-quantitative real time RT-PCR, ensured that quality requirements for our gene expression data were met. However, only the mRNA expression of 6 genes involved in folate metabolism and acquired resistance to 5-FU was addressed in this study. Greater insight could be gained by looking at more genes responsible for FA uptake, retention, metabolism and efflux. Additionally, since many of these enzymes are also post-translationally regulated, assessing protein expression would also contribute to our understanding of underlying mechanisms.
5.3 Future directions

The main aim of this study was to investigate whether FA supplementation modulates chemosensitivity of human breast cancer cells to 5-FU, where four different FA concentrations were chosen to parallel deficiency, mean and supplemental FA levels. Since the preferred culture medium for all three cell lines contains 2.3 µM FA, both 500 and 1000 nM FA concentrations may be deficiency models for these cell lines, despite being higher than reported physiologic serum FA concentrations found in humans. To address this limitation of the *in vitro* model, another *in vivo* study should be conducted, where FA intake can be controlled for and physiologically comparable serum and tissue folate levels could be attained relative to humans. Similarly to our study design, it is imperative that future studies are required to acclimatize animals to high FA diets in order to parallel chronic high FA levels in the North American population.

Due to the cell-specific variation in chemosensitivity to 5-FU we observed among the three cell lines, there is a need to test the chemosensitivity to 5-FU in a panel of breast cancer cell lines. Previous studies have demonstrated that cell lines display almost all of the genomic abnormalities including copy number and expression changes as the primary tumors [213]. Although all cell lines are classified either as basal like or luminal, responses to therapeutics are very heterogeneous and cannot be predicted by subtype alone [213]. Analyzing the effect of FA supplementation on chemosensitivity to 5-FU in as many distinct cell lines as possible, will allow for the identification of molecular events and mechanisms underlying the differences in 5-FU treatment we observed. This knowledge can be used as a platform for personalized targeted treatment formulations for breast cancer patients.
Further studies are required to help delineate the mechanisms underlying the effect of FA supplementation 5-FU chemosensitivity. This study only examined key enzymes in the folate pathway, TS, DHFR and MTHFR as well as MRPs associated with efflux. It may be of interest to examine the expression of RFC, PCFT and FRα as well as enzymes in 5-FU metabolism, uridine monophosphate kinase and uridine diphosphate kinase, responsible for the conversion of 5FdUMP to 5FdUTP that may play an important role in hereditary breast cancers that have BRCA mutations and poor prognosis. Similarly, due to the purported association between folate intake and ER expression, it might be of interest to examine how varying folate concentrations affected ER expression levels. An understanding of the mechanisms underlying the modulation of 5-FU chemosensitivity by FA supplementation will allow for improved study design in human studies, where confounding factors such as hormone status, alcohol consumption, BMI, dietary fat intake and alcohol consumption need to be adjusted for to capture the true effect of folate on 5-FU sensitivity [2].

Future in vitro studies should investigate the effect of supplemental FA using primary breast cancer cells that have not been immortalized and transformed. Currently, there are no studies in the literature that have assessed differences in the folate pathway and drug resistance to 5-FU among immortalized breast cancer cell lines and primary cancer cells. Furthermore, an in vivo xenograft model using human breast cancer cells would be informative, as it would be a better model for the human breast microenvironment than an in vitro model. A xenograft model would also likely better reflect human breast cancer compared to a carcinogen induced in vivo model, as most genetic aberrations are conserved from human breast tumors to cell lines.
Ultimately, this hypothesis should be tested in humans using a prospective cohort study design, where the cohort should be comprised of women diagnosed with invasive breast cancer and survival status would be the primary outcome measure. Participants should be interviewed about their FA containing supplement and/or multivitamin use, important dietary and lifestyle factors and medical history. Medical records should also be collected on tumor characteristics and treatment history. Having detailed records on subtype of breast cancer, type of chemotherapy treatment and duration, would capture the differential effect of FA on chemosensitivity to 5-FU that were observed in this in vitro study.

5.4 Conclusions

Our data are among the first to corroborate the inverse association between high intake and blood levels of FA prevalent in the North American population and reduced sensitivity to 5-FU in human breast cancer cells. Furthermore, our data indicate that FA supplementation influenced chemosensitivity of human breast cancer cells to 5-FU in a cell-specific manner; FA supplementation significantly decreased 5-FU chemosensitivity in MDA-MB 231 cells (p < 0.0001), significantly increased 5-FU chemosensitivity in HCC1937 cells (p=0.0059) and followed an inverted U shape in MCF-7 cells, whereby the lowest and highest concentrations of FA (5 and 1000 nM FA respectively) significantly increased chemosensitivity compared to 50 and 500 nM FA (p < 0.0001). LV increased chemosensitivity to 5-FU across all cell lines, where the effect was found to depend on medium folate levels. It is our hope that this data will serve as a platform for future mechanistic and human studies examining this relationship and help in the development of better health care recommendation for FA intake
and personalized treatment regimens tailored to the subtype of breast cancer in order to achieve maximal efficacy and minimal toxicity associated with 5-FU.
References


Wong, E. and J. Rebelo, Breast cancer pathogenesis and histologic/molecular subtypes in McMaster Pathophysiology Review.


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