January 2020

The Relationship Between Excessive Folic Acid Intake And Genome Instability In Human Lymphocytes

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THE RELATIONSHIP BETWEEN EXCESSIVE FOLIC ACID INTAKE AND GENOME INSTABILITY IN HUMAN LYMPHOCYTES

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2020

MAJOR: NUTRITION& FOOD SCIENCE

APPROVED BY:

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Advisor Date

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DEDICATION

I would love to dedicate this dissertation to my loving and supportive family

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Dr. Ahmad R. Heydari. I was fortunate enough to be his student and learn from his knowledge. Without his patience, support, and compassion, this dissertation would not have even been possible. I would like to thank my committee members, Dr. Ashok Bhagwat, Dr. Diane Cress, and Dr. Smiti Gupta, for their time and willingness to serve on this committee.

I want to thank Dr. Diane Cress for her support and kindness throughout my entire graduate journey. I was lucky to learn and gain knowledge from her during the master's mentorship. I would like to thank Andrew James for his support, kindness, assistance, and companionship through this journey.

Many thanks to lab members, Aiman Shahab, Dr. Manhel Albahri, my friends, Eno, Wala, Bayan, Meliani, Nouf, and fellow undergraduate students, Raiza, Iris, Helaina, and Hannah. I want to thank Raquel C., Riahana, and Chantae from GLML for their kindness and assistance.

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CHAPTER 1: BACKGROUND & SIGNIFICANCE

An optimal intake of dietary folate is essential since mammalian cells lack the enzyme required for folate biosynthesis [5]. Folate plays major roles in cell proliferation, protein synthesis, amino acid metabolism, and neurotransmitter synthesis, and all these functions of folate are achieved by tetrahydrofolate (THF) and its derivatives [6].

Folic acid (FA), or pteroylglutamic acid, is a fully oxidized compound which exists at an extremely low level in nature and was successfully synthesized by Angier and his colleagues in 1945 in an attempt to cure pernicious anemia [7]. As a provitamin, FA must be reduced in a two-step reaction by dihydrofolate reductase (DHFR) to be an active vitamin, i.e., the naturally occurring form of folate; 5-methyl-tetrahydrofolate (5-methyl-THF), and formyl-tetrahydrofolate (Formyl-THF) [1]. Upon activation to THF, FA can participate in the body folate pool as acceptor or donor of a methyl group in various pathways carried out by one-carbon metabolism, including DNA synthesis, DNA methylation and methionine regeneration. The common understanding that the majority of consumed FA is reduced to 5-MTHF emerged from early rodent studies, mostly rats, as well as studies on tumors and cell cultures. However, applying this concept to humans is very challenging. In addition to the high rate of enzymatic activity in rats, tumors and cell lines also exhibit high enzyme activity, perhaps due to supplementation of media with high FA concentration and high growth rate [3, 8]. In contrast, the activity of DHFR in human liver was discovered to be very low; only 2% of activity was observed in rat liver [3]. It appears that before the bioavailability of synthetic FA, the main physiological role of DHFR was to convert 7,8-dihydrofolate 7,8-(DHF) generated in the reaction of

thymidylate synthase (TS) to 5,6,7,8-THF (the active form) [9]. However, DHFR acquired another role to convert FA to 5,6,7,8-THF, i.e., FA is reduced to 7,8-DHF and then to 5,6,7,8-THF (**Fig 1.1**). DHFR activity is 850 times slower with FA as substrate than with 7,8-DHF in rats and 1300 times slower in humans [3]. Also, human DHFR activity was found to be 56 times slower with FA, and 35 times slower with 7,8-DHF compared to that of rats [3]. Furthermore, FA is not only a poor substrate for DHFR, but also a competitive inhibitor when 7,8-DHF concentration is high, and non-competitive inhibitor when 7,8-DHF concentration is low [3]. This phenomenon was also observed in rodents, human kinetics studies with recombinant DHFR [3, 10], and bacterial enzyme studies as well [3, 11].

FA is widely used in multivitamin supplements and is well known to improve folate status and cure anemia developed from folate deficiency [3, 12]. Moreover, FA supplementation reduces neural tube birth defects (NTD), and this finding has led several countries, including USA, Canada, and Chile, to implement mandatory fortification programs of food with FA in bread, cereal and grain products [12]. Despite the successful outcome in reducing NTD, overall 19-32% reduction [13, 14], and significant increase in population serum folate, many concerns have been raised about the safety and any unintended adverse outcome of exposing the entire population to high folic acid [15, 16]. Fortification programs aimed to increase the individual intake of folate to100-200 mcg/ day [12]. Nevertheless, several studies have reported that total folate intake over the upper limit (UL),1 mg/ day is now prevalent in the U.S [15]. Sacco et al. reported that children aged 1 to 3 years old exceed the UL intake in the U.S. [15] Besides other reports

that showed an increment level of plasma unmetabolized folic acid (UMFA)[12], a biomarker of excess FA intake[3] was found in consumers of supplements compared to non-consumers as a result of the US fortification program. UMFA appearance in plasma was found to be associated with an intake of 200 µg in a single dose, and several studies have linked UMFA to several adverse health outcomes [17]. Postmenopausal women who consumed a folate-rich diet and 400mcg/day of folic acid supplements had reduced natural killer (NK) cytotoxicity and higher UMFA levels compared to those who consumed lower supplementation and low-folate diet [18]. Sanchez et al. reported that high serum folate concentration (>45.3 nmol/L) was associated with increased DNA methylation of tumor suppressor gene *p*16 and DNA repair enzymes MLH1 and MGMT [19]. A shred of growing body evidence is indicating the relationship between FA supplementation, high FA intake and higher cancer onset and progression [16, 20], and increased mortality rate[16, 17, 21]. Furthermore, high FA intake can mask vitamin B12 deficiency and exacerbate adverse health outcome and cognitive impairment risk ratio in B12 deficient people [15-17].

The reported intakes exceeding the upper limit, either from fortified food alone or a combination of fortified food and supplements, will escalate the chronic exposure of several tissues to a high amount of unmetabolized FA. The low activity of DHFR in human liver and a high level of FA supplementation may result in functional folate deficiency, which would further escalate the deleterious effects associated with the presence of UMFA in systemic circulation in a certain populations [1, 17].

As more FA entering the cell through folate receptors (FRs) and/ or proton-coupled folate transport (PCFT), FA species will compete with 7,8-DHF and eventually saturate and inhibit DHFR, leading to the reduction of THF species, and accumulation of DHF which was also shown to be a potent inhibitor of methylenetetrahydrofolate reductase (MTHFR) in pig liver [22]. Thus, 5,10 methylene-THF would be spared for purine and pyrimidine synthesis leading to the decrease of 5-methyl-THF required for homocysteine re-methylation reactions (see proposed model **Fig. 1.2)**.

Folates, as other micronutrients, are suggested to have a U-shape effect with deleterious effects with both low and high concentration, especially with colon cancer [4]. Mason and Tang reported that excessive FA intake increases colorectal cancer risk, especially with the existence of precancerous lesions. [23].

An imbalanced diet, deficiency or excessive intake of some nutrients, is well known to affect the whole genome [8]. These effects lead to abnormal gene expression, chromosomal instability and eventually inherited mutations [24]. Micronuclei (MNi) scoring assay, an indicator of genome instability and genotoxic events, is found to be very sensitive to nutritional deficiency and excess. MNi frequency is found to increase rapidly with folate depletion *in vitro* [25]. Also, MNi frequency appears to be inversely associated with serum B12 in young females and positively associated with plasma homocysteine levels in young males [26]. Homocysteine is a functional marker for folate deficiency, while B12 is an essential cofactor for natural folate uptake and metabolism.

In this study**, we hypothesized that the chronic and excessive exposure of FA via fortified food consumption induces functional folate deficiency.** We proposed that

the consequent damage of this functional folate insufficiency at the cellular level resembles the DNA damage observed in folate depletion in human lymphocytes despite the normal systemic markers of folate status. **This hypothesis is tested for the following reasons:**

Specific Aims

Aim 1: To determine whether excessive FA intake in human lymphocytes induces functional folate deficiency *in vitro*. We hypothesized that excessive FA supplementation in human lymphocytes culture media induces DNA damage, similar to folate depletion. This damage is observed as an increase in micronuclei (MNi), nucleoplasmic bridges (NPB), and nuclear budding (NBUD) formation, cytome and DNA damage biomarkers associated with folate depletion. These markers are also known to be associated with events of DNA hypomethylation and impaired DNA repair mechanisms [24].

Aim 2: To determine whether high intake of FA through fortified food is associated with DNA damage in human lymphocytes in healthy adults. With increasing concerns about UMFA, we proposed that chronic exposure of FA through fortified food proposed induces functional folate deficiency and damage at the cellular level despite normal serum folate, RBC folate, and homocysteine levels in human subjects. We hypothesized that the high intake of FA is associated with high MNi, NPB, NBUD score frequency in human lymphocytes of healthy adults.

Aim 3: To determine the relationship between folate deficiency-driven genomic markers observed in human lymphocytes of healthy adults and traditional systemic folate status. Based on the proposed U-shaped relationship between folate

status and colorectal cancer specifically[23], there is an emerging need to find surrogate markers for folate status that would predict cellular damage earlier, especially when we look at some reports that indicated some tissues could be folate-depleted even though systemic folate markers indicate folate adequacy [27]. Thus, as a comprehensive phenotyping approach, the surrogate markers proposed for folate status such as MNi, uracil misincorporation, and LINE-1 methylation would be good candidates [6]. We hypothesized that there is a relationship between systemic markers and the genomic markers tested in this study. This relationship would help define the surrogate markers for folate status.

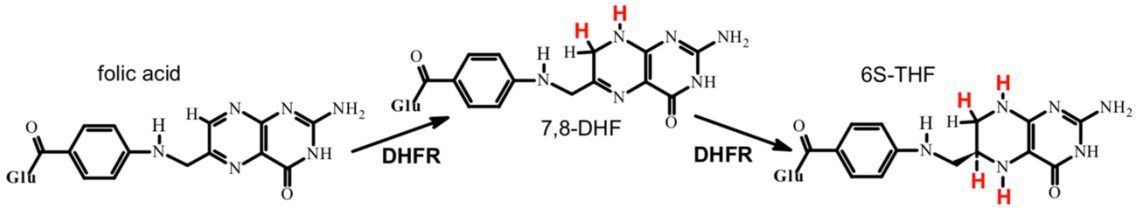


Figure 1.1: The Conversion of FA to active form tetrahydrofolate (THF). Two-step reduction of FA by DHFR. The oxidized pterin ring of FA requires the loss of more stabilization energy during the first reduction. V_{max} for DHFR with FA is extremely slower than with 7,8-DHF, regardless of the source of the enzyme. Glu, glutamate, [3].

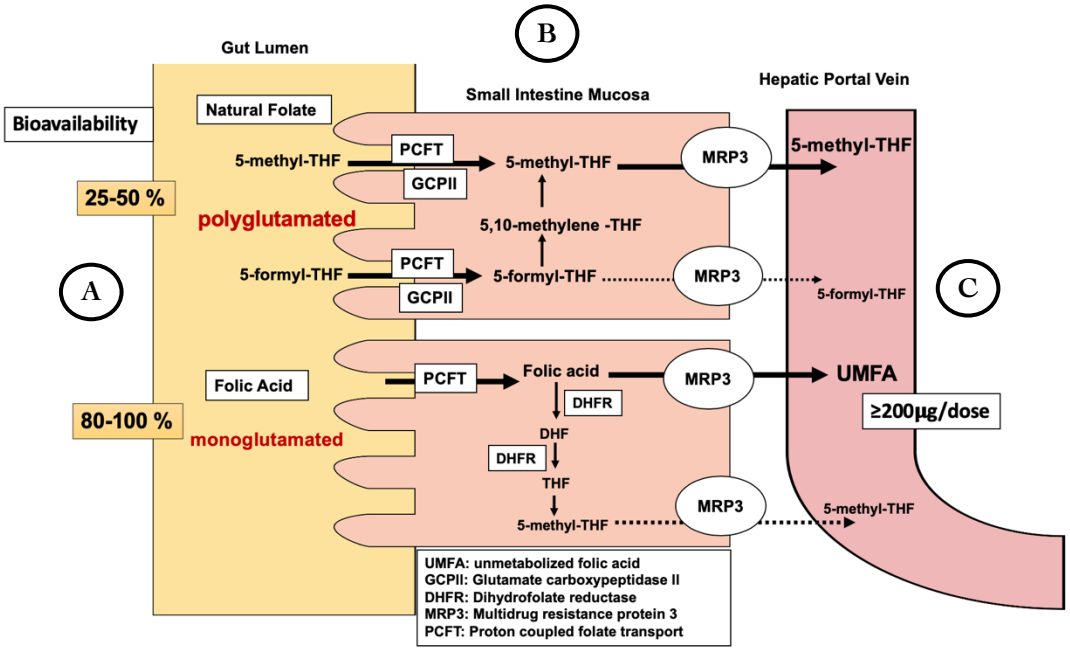


Figure 1.2 Folate and FA absorption by small intestine. A. Folate requires the removal of the polyglutamated chain by GCPII as it cross the cellular membrane and transport inside the cell by PCFT, which makes it less bioavailable than FA. **B**. Inside the intestinal mucosa, folate readily transports to blood or slightly modified as 5-methyl-THF (5-mTHF), whereas FA is required to be reduced twice by DHFR and methylated to be transported as 5-mTHF. **C.** Most absorbed folate appears in the blood as 5-mTHF, while most absorbed FA appears in the blood as unmetabolized FA (UMFA), usually associated with ingestion of 200µg FA per dose. (the dotted arrow indicates a small quantity present in the blood [1]).

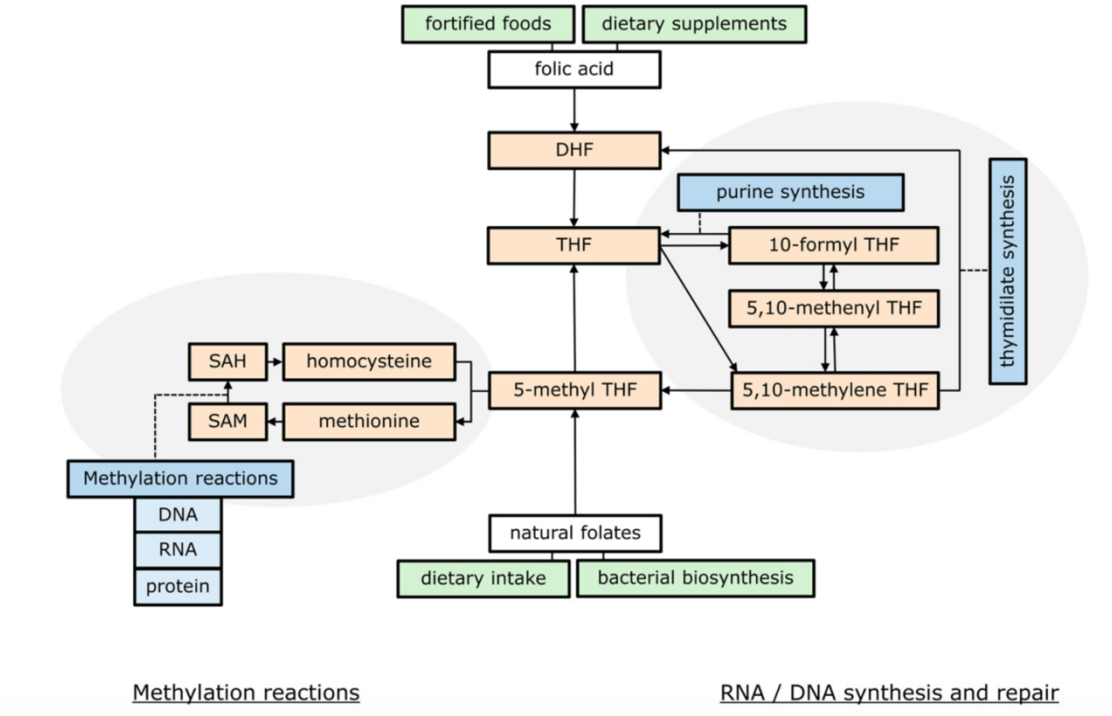


Figure 1.3: Folate and FA metabolism pathways. Folate enters the cell as 5-mTHF and donates the methyl group for homocysteine re-methylation reaction before participation in the folate cellular pool. FA enters the cell as UMFA and requires reduction by DHFR before participation in the cellular folate pool [*4*].

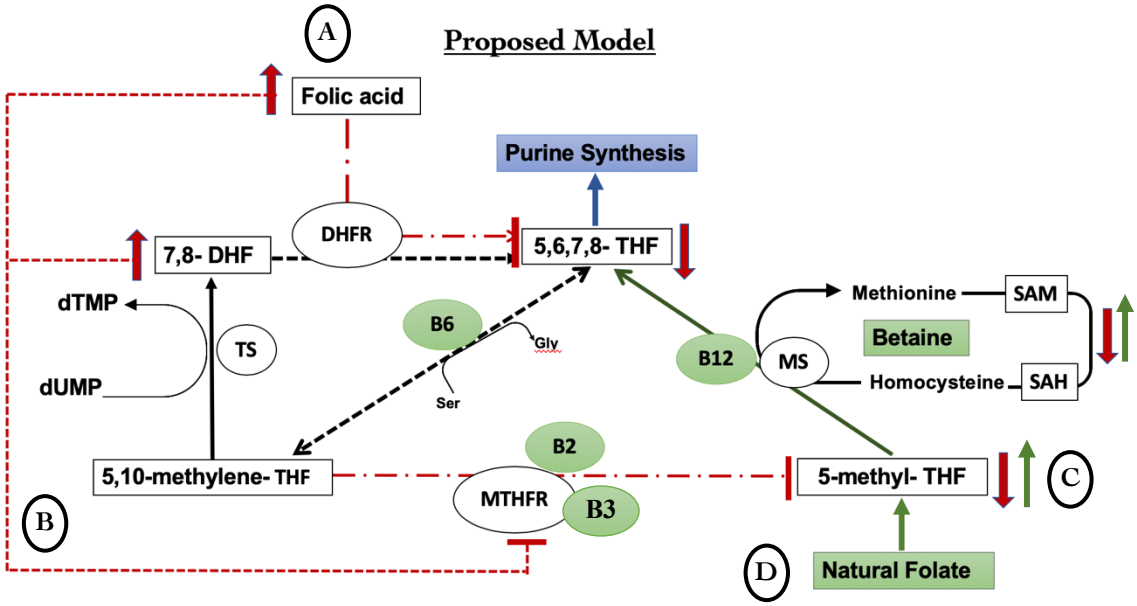


Figure 1.4: Schematic depiction of the proposed model of the study. A. As high amount of FA molecules enters the cells, they compete with DHF molecules for DHFR. FA is a poor substrate and potent inhibitor for DHFR, so THF concentration decreases. **B**. The accumulation of FA and DHF send negative feedback to MTHFR to terminate the conversion of 5,10-methylene-THF to be preserved for nucleotide synthesis. **C.** As a result of MTHFR inhibition, less 5-mTHF is available for methylation reactions (red arrows). **D.** Folate species can mitigate the effect of FA by donating the methyl group for methylation reaction and then participating in nucleotide synthesis (green arrows). thymidylate synthase (**TS**), methionine synthetase (**MS**).

CHAPTER 2: THE IMPACT OF SUPRAPHYSIOLOGICAL CONCENTRATION OF FOLIC ACID ON GENOME STABILITY OF HUMAN LYMPHOBLASTOID CELL LINE *IN VITRO*

2.1 Abstract

Folate metabolism is pivotal for many cellular processes, including cell proliferation, nucleotide biosynthesis, methionine regeneration, and epigenetic regulation. Folate deficiency has been linked to several diseases, including neural tube defects (NTD), cancer, and chromosome aneuploidy, such as Down syndrome. Several studies suggested that folic acid (FA), a synthetic version of natural folate, has a U-shaped effect on health and disease. Since FA was introduced into our diets in 1998 after mandatory fortification of grain and grain products, several opinions have raised concerns about its safety and toxicity level for humans. In this study, we evaluated the impact of high FA intake (supraphysiological level) on the genome stability of human lymphoblastoid *in vitro*. By utilizing CBMN assay, we found high concentrations of FA in media; media deficient in FA resulted in a statistically significant increase in cytome biomarkers in LCLs cells; interestingly, a U-shaped trend is observed between FA concentrations and cytome biomarkers.

2.2 Introduction

Folate is a pivotal nutrient for mammalian cells that plays a fundamental role in DNA metabolism. It is required for the synthesis of dTMP from dUMP and S-adenosyl methionine (SAM) from methionine. There is accumulated evidence that under the condition of folate deficiency, dUMP accumulated, leading to uracil incorporation into DNA in place of thymine. Excessive uracil misincorporation into DNA may generate

point mutation, single and double-strand breaks, and chromosome breakage[8, 28, 29]. In addition to the deleterious effect of uracil, folate (5-mTHF) is required for a constant supply of methionine through the conversion of homocysteine in a B12-dependent reaction. Therefore, a decrease in methionine regeneration leads to a decline in SAM synthesis, a methyl donor essential for several cellular and DNA methylation reactions, which in turn leads to alteration of the DNA methylation pattern, gene expression, and eventually chromosome aberration [8, 30].

Since humans, as other mammalians, lack the enzyme for folate synthesis, obtaining folate from a well-balanced diet is crucial. A typical western diet content of natural folate can easily fall below the recommended intake (400 mcg/day) [31], especially in low socio-economic population [32, 33]. Low serum and RBC folate in women of childbearing age are associated with an increase in NTD [34, 35], which has led several countries including USA to fortify grain and grain products with Folic acid (FA), a synthetic stable version of folate[12]. However, despite the similarity in structure, FA is handled differently than the natural form. Unlike folate, FA is required to be activated into a two-step reduction by dihydrofolate reductase (DHFR) [36] and then can participate in the folate pool for thymidylate and nucleotide synthesis. Further, the action of methylenetetrahydrofolate reductase (MTHFR) is required to be converted into 5-mTHF, the predominant form in the blood, in B2 dependent reaction [6]. Furthermore, Patanwala et al. showed that most absorbed FA appears in the blood as unmetabolized FA (UMFA)[1], which is also associated with the ingestion of a diet containing more than 200 µg of FA [17]. UMFA is linked to several health issues, including reduced

natural killer cytotoxicity in postmenopausal women [18] and in mice models [37]. The presence and persistence of UMFA in the blood are thought to be the result of low DHFR activity in general in human, as well as the inhibition effect of FA to DHFR. FA poses a fully aromatic pteridine ring that imposes a greater barrier for DHFR than DHF. Also, FA was found to be either a competitive inhibitor or an uncompetitive inhibitor based on cellular DHF concentration. DHFR slow activity and inhibition effect with FA is not only found in humans but also in rodents [3]. The common understanding that the majority of consumed folic acid is reduced to 5-MTHF emerged from early rodent studies, mostly rats, as well as studies on tumors and cell culture. This common belief led most publications to use FA and folate interchangeably, then furthering confusion between the benefits and consequences of both forms. Natural folate is less stable than FA, so it is unlikely to consume a high amount of natural folate. However, due to its stability, FA upper limit (UL) intake was set at 1000 µg DFE, a level at which FA can conceal B12 deficiency [6, 38, 39].

Folate deficiency can lead to elevated DNA damage and DNA hypomethylation, which are both risk factors for cancer [40, 41]. However, several opinions have increased Skepticism over the beneficial effects of high intake of FA (synthetic version), and its deleterious effect in cancer progression, especially in colon cancer, which is mostly linked to imbalanced folate status[16].

Folate, like other micronutrients, is suggested to have the so-called U-shaped effect with deleterious effects with both low and high concentrations. Mason and Tang reported that excessive folic acid intake increases colorectal cancer risk, especially with

the existence of precancerous lesions[23]. In this study, we evaluated the impact of high FA intake (supraphysiological level) on the genome stability of human lymphoblastoid *in vitro*.

2.3 Materials and Methods

Cell culture

Human lymphoblastoid cell lines (LCLs) were obtained from Coriell cell repositories. LCLs were cultured in RPMI-1640 (free FA) supplemented with 10% dialyzed FBS, 1% penicillin/ streptomycin (5000 IU penicillin/ 5 mg streptomycin), 1% glutamax and 1% sodium pyruvate and incubated in a humidified atmosphere with 5% CO_2 at 37° C. Cells were initially grown in a medium containing a final concentration of 300 nM FA for three passages before seeding cells in either 12, 180, 300, 2300, and 10,000 nM FA, as shown in study design (1), **Fig 2.1**.

Doubling Time

We calculated the population doubling time (DT), or the time required for a culture to double in number, using the formula: DT=In2/In (Xe/Xb). T: the incubation time in any units, Xb: the cell number at the beginning of incubation time, Xe: the cell number at the end of the incubation time. Cells were seeded at three different concentrations in a 12-well culture plate, and counted after 4hr, as 0 hr, and then for 24 hr, 48hr and 72hr using trypan blue and an automated cell counter, TC20 ™ (Bio-Rad, USA). DT is reported as the mean of 3 days.

Homocysteine Assay

Cells were washed twice with ice-cold 1X PBS and then collected by centrifugation at 2000 x g for 10 minutes at 4°C. The cell pellet was homogenized on ice in 1mL ice-cold 1X PBS and centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was removed and stored on ice. The homocysteine level was determined by a commercially available homocysteine ELISA kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instructions.

LINE-1 Methylation Assay

Genomic DNAs were isolated using PureLink® Genomic DNA Mini Kit (Life Technologies, Carlsbad, CA), following the manufacture's protocols. LINE-1 methylation assay was performed using the Global DNA Methylation LINE-1 kit (Active Motif, Carlsbad, CA). Briefly, 100 ng MseI digested genomic DNA was hybridized with LINE-1 probe and immobilized to a streptavidin-coated plate. After binding of primary and secondary antibodies and setting colorimetric reaction, data were obtained and analyzed using a standard curve of methylated and non-methylated DNA.

Cytokinesis-block Micronucleus (CBMN)Assay

We followed the protocols of Thomas and Fenech (2011) [42]. Briefly, on the day of assay, cells were washed twice in Hanks balanced saline solution (HBSS) and then incubated in supplemented RPMI-1640 medium containing either 12, 180, 300, 2300, and 10000 nM final concentration of FA and at a final concentration of 4.5 μg/ml cytochalasin B (Sigma Aldrich). After 24 hours, cells were harvested in duplicate using Cytospin 4 (Shandon) at 600 rpm for 5 min. Slides were air-dried, fixed, and stained using Shandon

Kwik-Diff Stains (Thermo Scientific). Slides were cover-slipped using DPX Mountant (Sigma Aldrich). The frequency of MNi, NPB, and NBUD (cytome biomarkers) was determined in 2000 binucleated (BN) cells following the scoring criteria of HUMN project guidelines[43]. Slides were coded and scored by two trained scorers who had no access to the codes. Cytome biomarker scores were presented per 1000 Binucleated cells (BN).

Exposure of LCLs to hydrogen peroxide and CBMN assay

We followed the protocols by Main et al. (2013)[44]. Briefly, on day 9 of the assay outlined in **Fig 2.6**, cells were washed twice in HBSS. Then, cells were exposed in RPMI-1640 medium supplemented with a final concentration of 100 µM hydrogen peroxide for one hour. The cells were then washed again in HBSS and resuspended in RPMI-1640 cell culture media containing either 180, 300, and 2300 nM final concentration of FA before exposure to CB to complete CBMN assay, as described earlier.

Gene Expression Analysis

The mRNA expression level of various genes was quantified using quantitative real-time PCR (qPCR), PikoReal 96 (Thermofisher, Vantaa, Finland). Total RNA was extracted from LCLs using TRIzol® Reagent (Gibco BRL, Rockville, MD). The isolated mRNA was reverse transcribed using and following the protocols ImProm-II™ Reverse Transcription System. Transcript of each gene was normalized to the geometric mean of HPRT1 and ß-Actin. External standards for each gene were prepared by subcloning using the TOPO® TA Cloning® kit (Invitrogen, Carlsbad, CA).

Statistical Analysis

Results were presented as mean (± SEM) and analyzed using one-way ANOVA for comparison between groups. *Post hoc* student t-test was applied for comparison between two groups using the software MICROSOFT ®EXCEL, version 16. *P*-value < 0.05 was considered statistically significant.

2.4 Results

We evaluated genome instability in response to a wide range of FA concentrations so that we were able to look into the U-shaped association. We determined five different concentration from previously published studies, where 10,000 nmol/L represents the supraphysiological level [17, 45], 180-300 nmol/L represents proposed optimal physiological level [8, 46, 47], and 12 nmol/L represents the depletion level [8, 17]. Also, 2300 nmol/L will be included as the level supplementation at which routine media generally used in cell culture practices.

Doubling Time

Generally, the DT of LCLs ranges between 18hrs to 36 hrs and is a good indicator of the normal proliferation rate of these cells. While the average DT of cells grown under conditions of 300, 2300 and 10,000 nM was 36 hrs, cells grown under 180 nM experienced a longer time, $P<0.05$. FA-deficient LCLs (12 nM), as expected, encountered significantly extended DT, $P<0.05$, i.e., reduced proliferation rate.

Homocysteine level

Homocysteine is a well-accepted marker as a functional indicator for folate deficiency, yet it is not specifically a reflection of folate status. We evaluated the impact

of high FA (10,000 nM) on LCLs and compared it to other proposed concentrations, including depletion level (12nM). Surprisingly, we found no statistically significant difference across different FA treatments, though, a trend of a U-shaped association was observed.

LINE-1 Methylation Level

Folate deficiency is associated with an alteration in the methylation level of a long interspersed nuclear element (LINE-1), a 64% lower methylation level. These mobile parasitic genetic elements comprised 17% of the human genome, and their methylation level is considered to be a surrogate marker of global genomic DNA methylation. We investigated the impact of high FA intake on the LINE-1 methylation level. As shown in **Fig.2.4**, there was a statistically significant decrease in methylation level in response to both the supraphysiological level (10,000nM) and deficiency level (12nM) of FA. Though the level of hypomethylation was variable between different LCLs, hypomethylation was more prominent at the depletion level. However, the global methylation level of LINE-1 did not differ significantly between 180,300,2300 nM.

Cytome Biomarkers scores in response to different FA concentration

It is well documented that FA deficiency induces DNA damage that can be measured by CBMN assay. This assay measures endpoints DNA damage, such as micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss; Nucleoplasmic bridge (NPB), a biomarker of DNA misrepair and/or telomere end-fusions; Nuclear bud (NBUD), a biomarker of elimination of amplified DNA and/or DNA repair complexes. Crott et al. showed that a minimum of 120 nM of FA is required

to lower the formation of MNi, NPB, and NBUD (cytome biomarkers) in primary human lymphocytes. Our results indicated that in response to high FA concentration in media and media deficient in FA, there was a statistically significant increase in Cytome biomarkers in LCLs when compared with 180,300 and 2300 nM. Interestingly, a U-shaped trend was observed between FA concentration and cytome biomarkers in human LCLs, as shown in **Fig 2.5**. Also, 300nM of FA concentration encountered the lowest MNi and NPB scores when compared with 180 and 2300 nM of FA, $p<0.05$.

Cytome biomarker scores in response to different FA concentration after exposure to hydrogen peroxide

Further, we wanted to compare among 180, 300, and 2300 nM of FA concentration in terms of repair capacity after exposure to hydrogen peroxide, i.e., oxidative stress repair response. As shown in **Fig. 2.6**, we scored cytome biomarkers after hydrogen peroxide exposure in 24- and 72-hour recovery periods for 300nM of FA and only 72-hour recovery periods for 180, and 2300 nM of FA. The reason for this approach was to test how vast cytome biomarkers differ when cells were incubated at both lower and higher concentrations than the concentration at which the lowest cytome biomarkers scored in our study, 300 nM FA, **Fig 2.5**. Our results, **Fig 2.7**, showed that at 300 nM (T_300), there was no statistical difference between 24- and 72-hour incubation periods in terms of MNi and NPB scores. However, compared to 300 nM, MNi and NBUD scores were higher at 180 nM (T_180) and 2300 nM (T_2300) when incubated at 72 hours after H_2O_2 exposure, $p<0.05$.

Gene expression analysis

High concentrations of FA (10,000 nM) in media showed a similar impact on the gene expression level of tumor suppressor genes (p16 and p21), cell cycle checkpoint gene (RAD21), and BER genes, as seen in **Fig 2.8**. Uracil is removed by uracil-DNA-glycosylase 2 (UNG) and replaced with thymidine by DNA polymerase beta (ß-POL). This impact was similar to FA deficiency (12 nM) where UNG and ß-POL expression levels were higher than 180, 300, 2300 nM, $p<0.05$, but not significant for RAD21, p16, and p21.

2.5 Discussion

In this study, two important outcomes emerged. First, the supraphysiological concentrations of folic acid in media appeared to be genotoxic for LCLs *in vitro*. Micronucleus index, utilized by CBMN assay in human cells, is one of the standard cytogenetic and genetic toxicology tests [48]. MNi are derived from events that lead to chromosome fragments or whole chromosome to lag behind anaphase throughout the nuclear division[49]. In evaluating the cytome biomarkers, we saw a prominent increase in MNi scores at 10,000 nM compared to 180, 300, 2300 nM FA, in **Fig 2.5**. To our knowledge, no previously published studies have evaluated the toxic impact of the supraphysiological level of FA concentration in media on human LCLs, i.e., MNi scores. Considering the U-shaped association, the impact of high FA concentrations could be similar to a deficiency level (12nM), indicating a functional deficiency, yet the underlying mechanisms are yet to be known. However, by evaluating the effect of high FA concentration on global LINE-1 methylation (**Fig 2.4**) and UNG-BER genes (**Fig 2.8**

A&B), we can speculate that the increment in MNi formation could be linked to genomic hypomethylation and uracil misincorporation events. The link between uracil incorporation into DNA and the consequent generation of double-strand breaks (DSB) was established by multiple studies. Constant removal of uracil by UNG, followed by incomplete repair of generated gaps by ß-POL, lead to DSB, chromosome breakage and chromosome loss[50, 51], which eventually would be eliminated from the nucleus as MNi expressed in the cytoplasm[2, 48, 49]. We saw an increase in UNG and ß-POL gens expression levels, but this finding needs to be further confirmed with protein expression analysis, uracil quantification assay [52] or UNG-BER assay. Nonetheless, our RAD21 gene expression level results did not show a significant difference across the five different concentrations (**Fig 2.8 E**), though a trend was observed. RAD21 was shown to involve in cellular S-phase arrest and consequent repair of DSB by homologous recombination (HR) and sister chromatid cohesion[53]. It was suggested that DSB induced by FA deficiency is unlikely to be repaired by HR in which BRCA1 and BRCA2 genes play an important roles [8, 54]. This drives the importance to investigate (**a**) whether DSBs are induced by high FA concentrations in LCLs, and (**b**) whether the misrepair of DSBs is an underlying mechanism of MNi formation in response to high FA concentrations. Further experiments using comet assay and y-H2AX are required. Henry et al. showed that both super FA (10 mg/kg) and FA deficient diets (0.1 mg/kg) for 5 months had a similar impact on mice (succinylsulfathiazole treated), where similarly both had compromised nucleotide metabolism, pyrimidine metabolism, low lymphocytes number, especially B-cells[55]. In addition to uracil misincorporation, hypomethylation of DNA

has been linked to folate deficiency and MNi formation. Hypomethylation of CpG island leads to heterochromatin defects, centromere instability, and chromosome malsegregation and eventual loss as MNi[8, 24, 49]. Our data showed that hypomethylation was induced when cells incubated in high concentrations of FA as well as in FA deficient media, i.e., low LINE-1 methylation level (**Fig 2.4).** The hypomethylation of LINE-1 pattern across the five FA concentrations was similar between different LCLs (listed in **Table 2.1**), with a degree of variation. These results support the finding of Charles et al. that the supraphysiological level of FA reduced LINE-1 methylation level significantly in human lung fibroblast and colon epithelial cell lines, and the impact was passage dependent [45]. The hypomethylation effect induced by the supraphysiological level of FA could be explained by the limited capacity of cellular DHFR and MTHFR to reduce FA to 5-mTHF, the active form required for methionine regeneration and SAM synthesis. Even though the DHFR activity *in vitro* is over more expressed in human liver tissue [56], the LCLs capacity to handle FA in long term incubation is unknown. It was reported by Christensen et al. that FA supplementation led to a significant decline in mRNA expression and protein activity of MTHFR in mouse models. The decline of MTHFR activity effectively reduced 5-mTHF concentrations in high FA-fed mice compared to controls [57]. It was also reported that FA supplementation leads to the inhibition of MTHFR in crude brain extract [58], as well as in crude liver extract [57], suggesting that UMFA could lead to MTHFR deficiency. Few studies have investigated the impact of high FA concentrations on different human tissues *in vitro*, so further studies are required.

The second outcome this study showed was that moderate folate deficiency has a strong effect on the genome stability of LCLs once exposed to genotoxic agents, such as hydrogen peroxide. We showed that MNi and NBUD formation increased significantly in cells exposed to hydrogen peroxide and incubated for 72 hour in 180 and 2300 nM FA-media compared to 300 nM FA-media, **Fig 2.7**. This could indicate that folate status impairs DNA repair response and/or interferes with the cytotoxic cellular response. Thus, finding the optimal concentration to grow LCLs is very important for proper research planning and data interpretation. Based on our results, 300nM appears to be an optimal concentration of FA in media for LCLs with respect to proliferation rate, homocysteine level, cytome biomarkers, and expression of stress response genes.

Given together our results, the supraphysiological concentrations of FA in LCLs media-induced negative impacts on genome stability *in vitro* in a pattern appeared to be similar to those of FA deficiency. A U-shaped association was observed in terms of cytome biomarkers in response to FA status. The findings of this study support the concerns raised by others about the safety of prolonged exposure to excessive amounts of FA in the diet through fortified food.

Study Design (1):

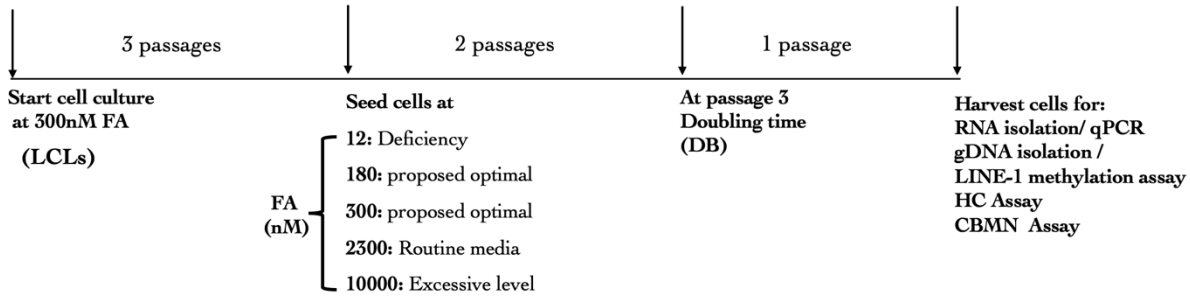


Figure 2.1: Schematic diagram of study design (1). An outline of the long-term culture and assays tested the cytotoxic and genotoxic effects of supraphysiological concentration of folic acid was compared to deficiency, proposed optimal, and routine media level on LCLs. FA, folic acid, HC, homocysteine, gDNA, genomic DNA, CBMN, cytokinesis blocked micronucleus assay.

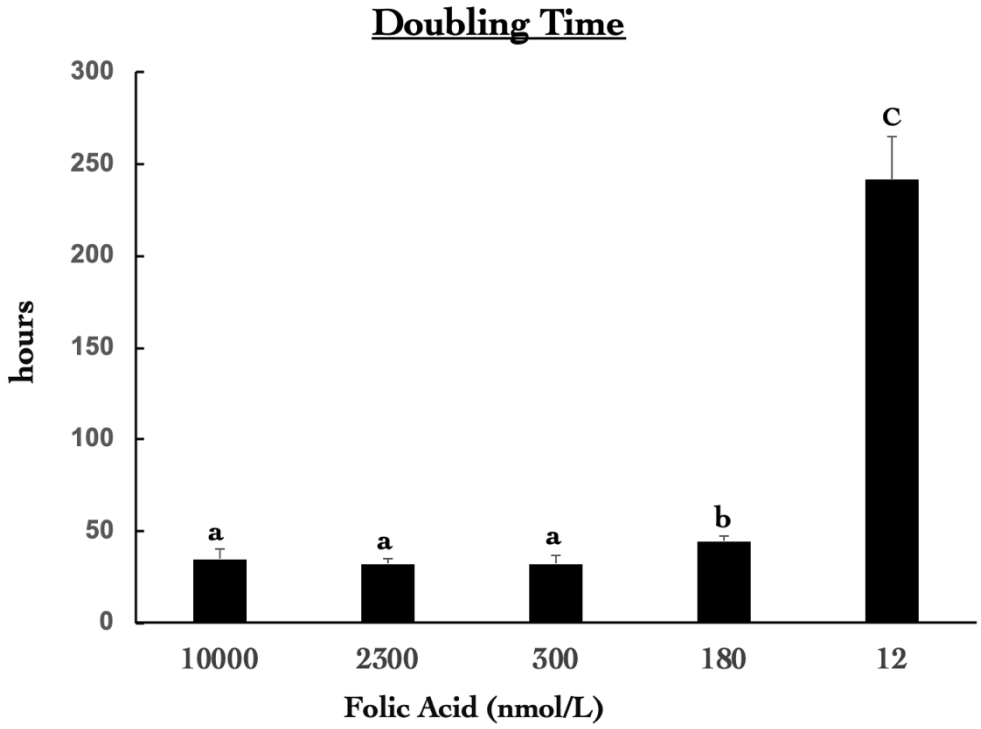


Figure 2.2: Doubling time of human LCLs in response to different FA concentrations. LCL (GM16113). Data were presented as mean (± SEM), n=3. Values with different superscripts indicate significant differences at $p<0.05$. ANOVA $P<0.001$.

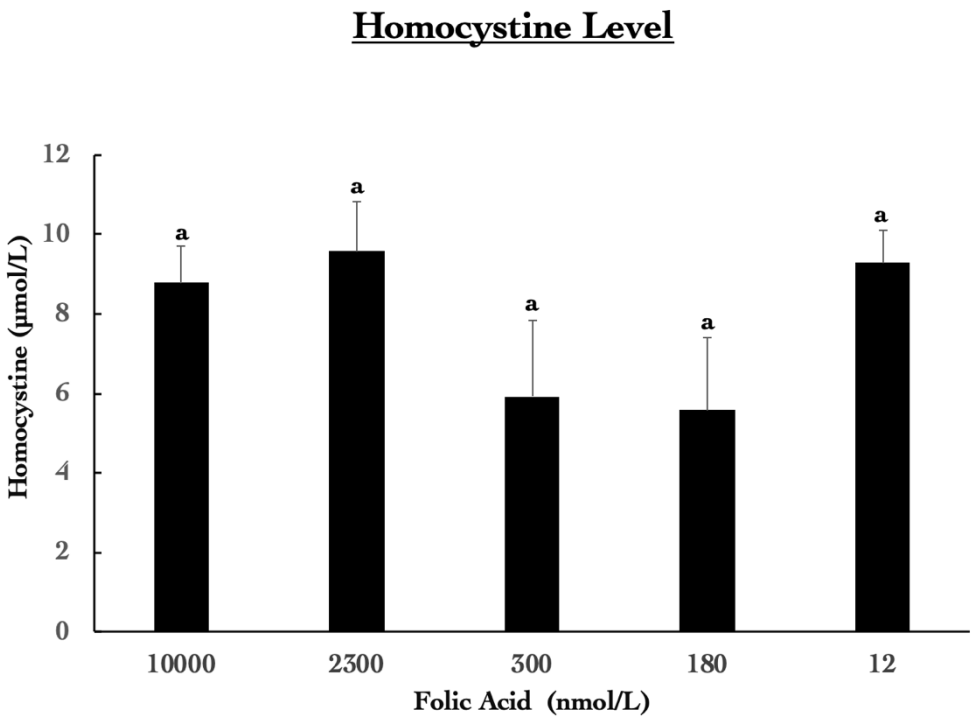


Figure 2.3: Homocysteine levels in response to different FA concentrations in human LCLs. LCL (GM16113). Data presented as mean (± SEM), n=3. Values with different superscripts indicate significant differences at $p<0.05$. ANOVA *P*= ns.

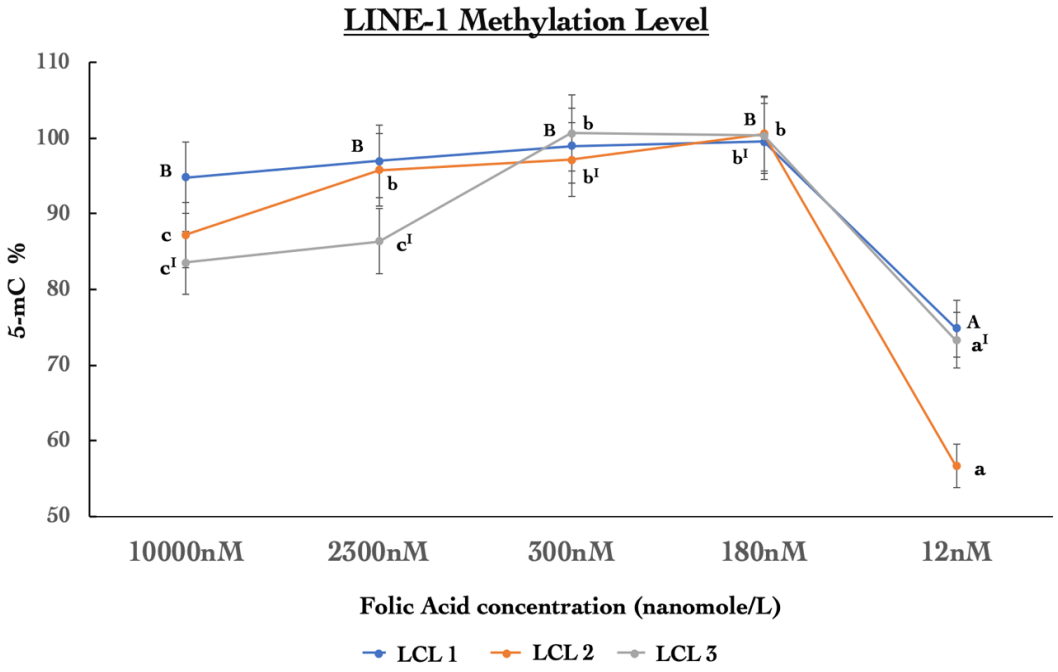


Figure 2.4: LINE-1 methylation levels in response to different FA concentrations in human LCLs. The level of 5-mC % associated with detectable CpG residues was measured by LINE-1 methylation assay. LCL 1 (GM16118), LCL 2 (GM16113), LCL 3 (GM00130). Data presented as mean (± SEM), n=3. Values with different superscripts indicate significant differences at $p<0.05$. ANOVA $P<0.001$

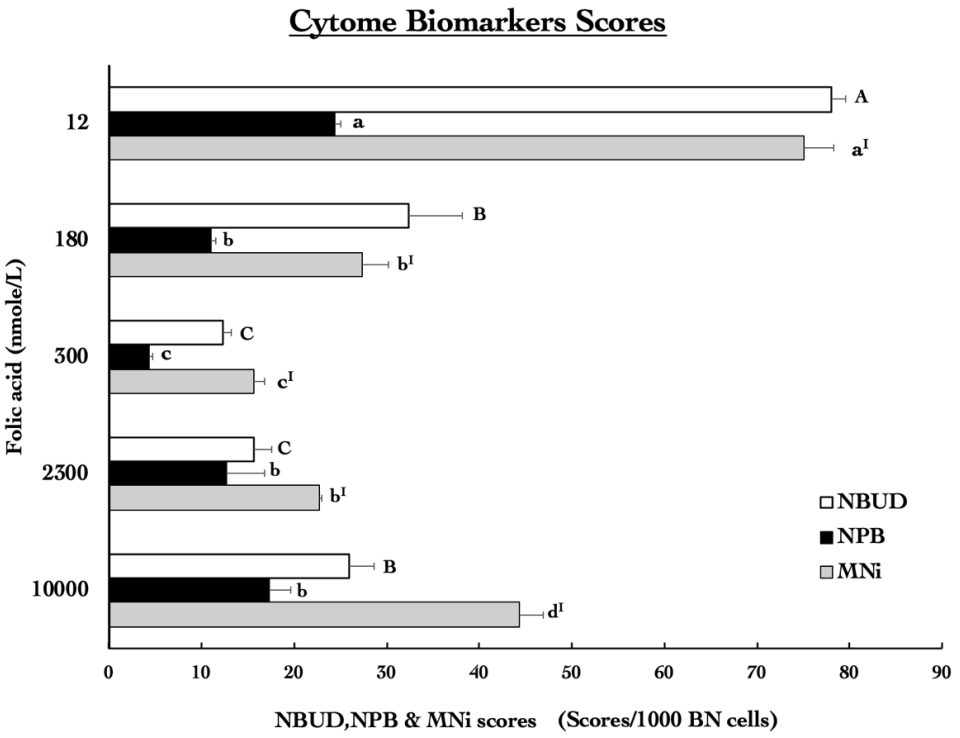


Figure 2.5: Cytome biomarker frequency scores in response to different FA concentrations in human LCLs. MNi, NP & NBUD scores were measured by CBMN assay as of study design (1). Scores represented per 1000 binucleated (BN) cells. LCL (GM16113). Data presented as mean (± SEM), n=3. Values with different superscripts indicate significant differences at $p<0.05$. ANOVA $P<0.001$.

Study Design (2) :

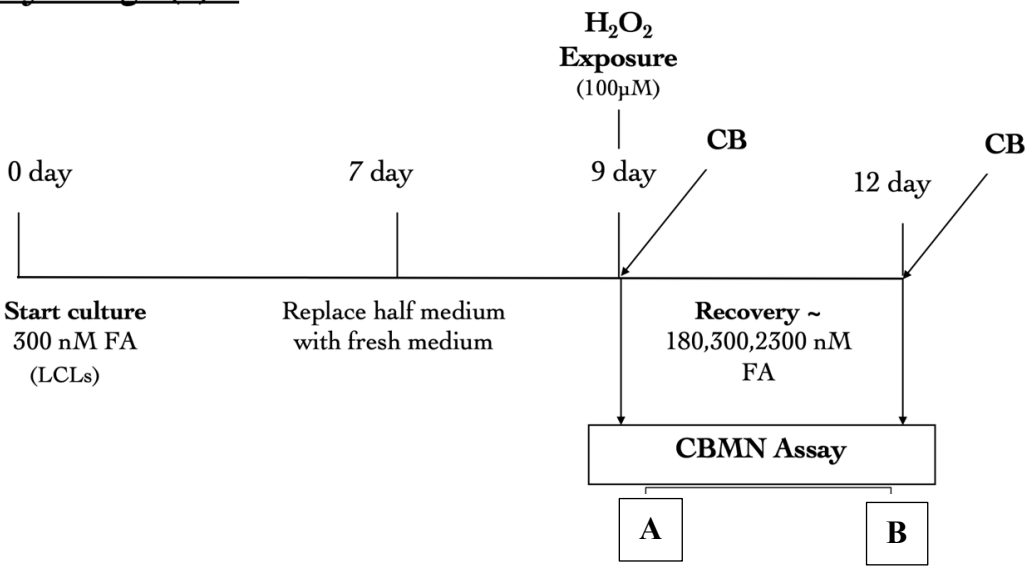


Figure 2.6: Schematic diagram of Study design (2). An outline of the long-term culture and CBMN assay tested the cytotoxic and genotoxic effects of hydrogen peroxide (H_2O_2) on LCLs, (GM16113). CB: cytochalasin-B. **A:** scores for C_300, and T_300 (24 hr.) were collected. **B:** scores for T_300, T_180, and T_2300 (72 hr.) were collected.

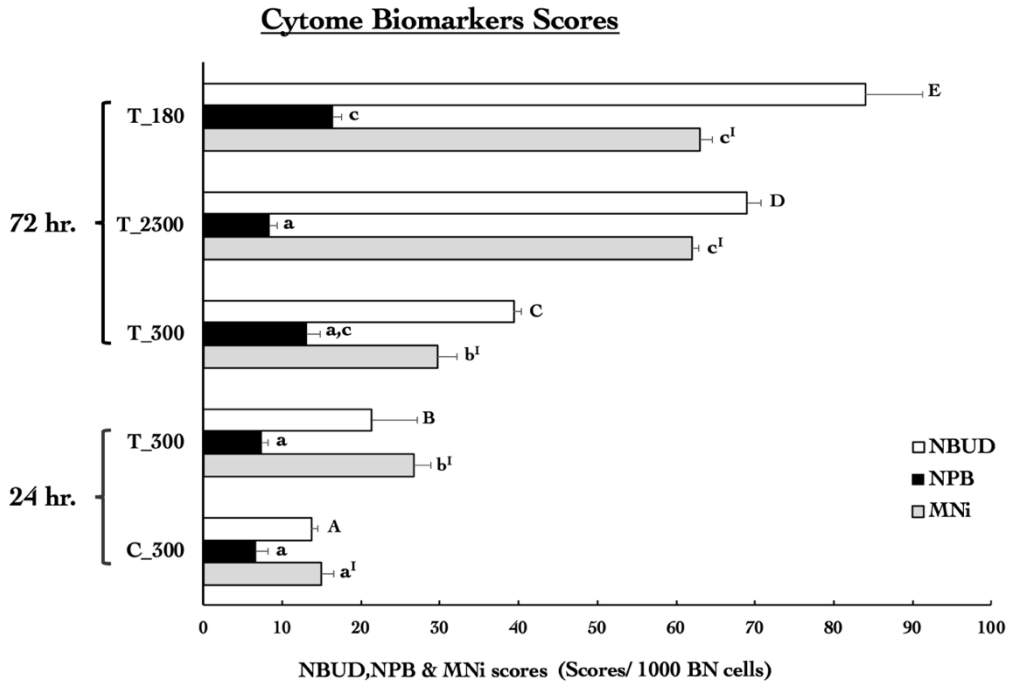
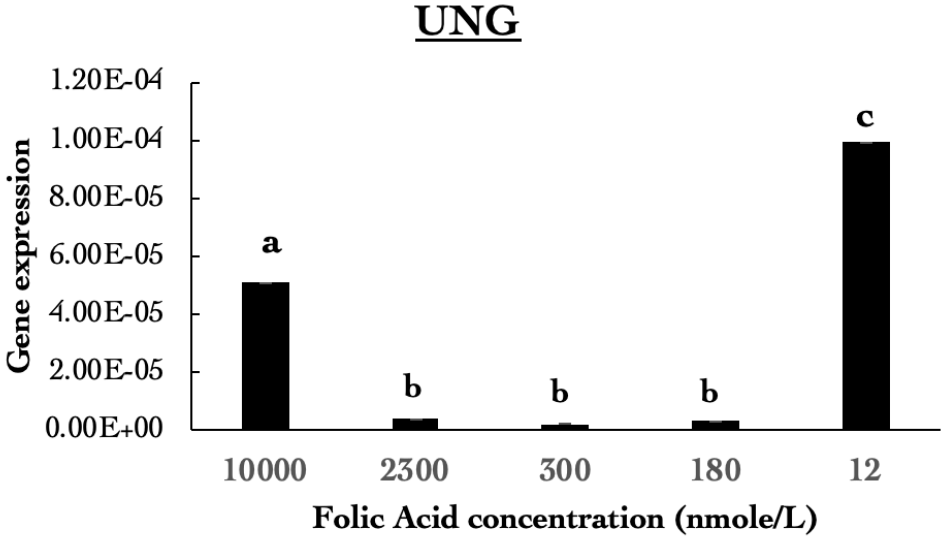
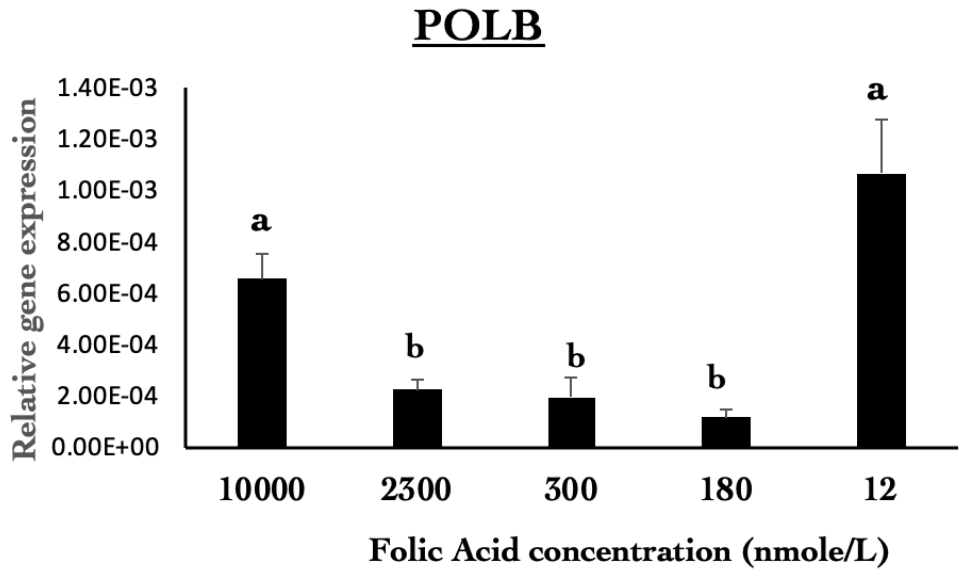


Figure 2.7: Cytome biomarkers frequency scores in response to different FA concentrations in human LCLs. MNi, NP & NBUD scores were measured by CBMN assay as of study design (2). Scores represented per 1000 binucleated (BN) cells assay. **T**: treatment, **C:** control, **180:** 180 nM, **300:** 300 nM and **2300:** 2300 nM (folic acid concentration). LCL (GM16113). Data presented as mean (± SEM), n=3. Values with different superscripts indicate significant differences at $p<0.05$. ANOVA $P<0.0001$.

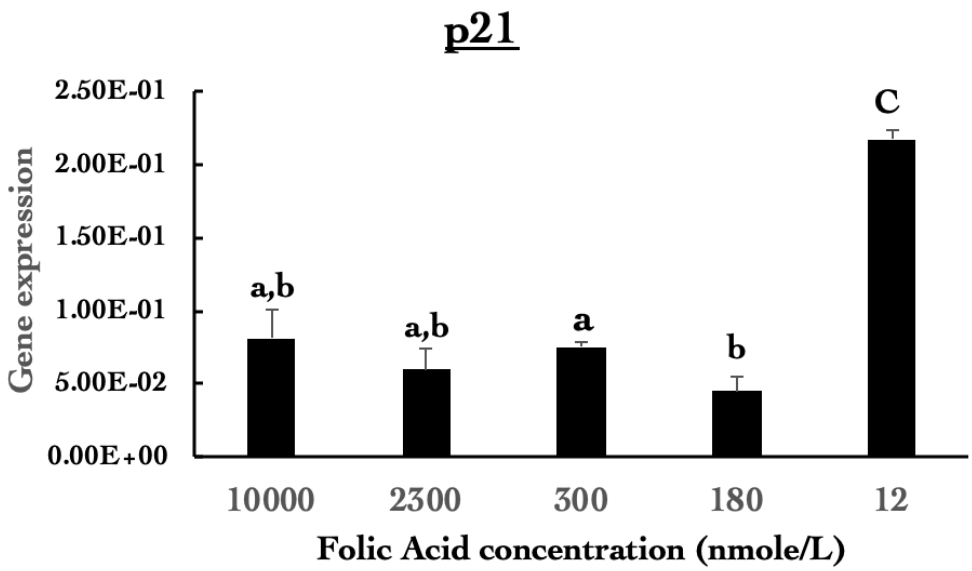
A


UNG
1.20E-04
1.00E-04
8.00E-05
6.00E-05
4.00E-05
2.00E-05
0.00E+00
Gene expression
a
b
b
b
c
10000
2300
300
180
12
Folic Acid concentration (nmole/L)

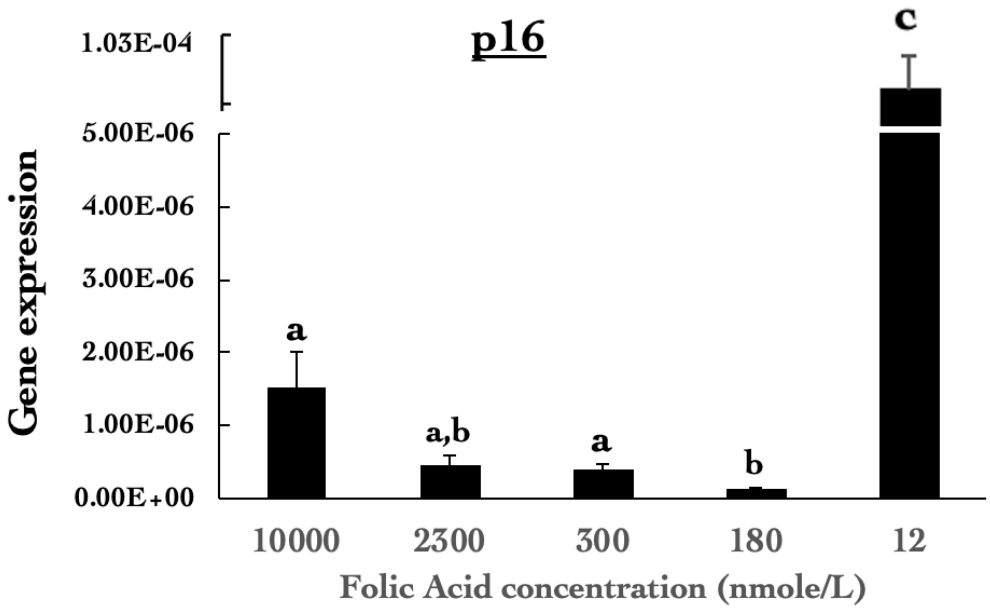
B


POLB
1.40E-03
1.20E-03
1.00E-03
8.00E-04
6.00E-04
4.00E-04
2.00E-04
0.00E+00
Relative gene expression
a
b
b
b
a
10000
2300
300
180
12
Folic Acid concentration (nmole/L)

C


p21
2.50E-01
2.00E-01
1.50E-01
1.00E-01
5.00E-02
0.00E+00
Gene expression
a,b
a,b
a
b
C
10000
2300
300
180
12
Folic Acid concentration (nmole/L)

D


p16
1.03E-04
5.00E-06
4.00E-06
3.00E-06
2.00E-06
1.00E-06
0.00E+00
Gene expression
a
a,b
a
b
c
10000
2300
300
180
12
Folic Acid concentration (nmole/L)

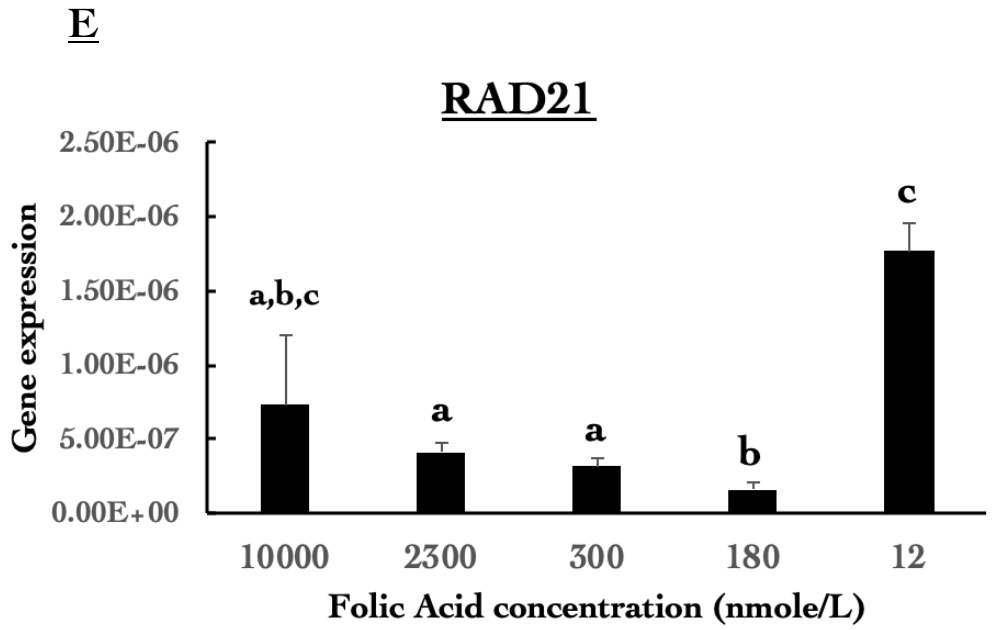


Figure 2.8: Gene profiling in response to different FA concentrations. A-E. Gene expression was evaluated in LCLs (GM16113) grown in triplicate. Transcript levels were determined using RT-qPCR and normalized to geometric mean of ß-Actin and HPRT1. **A.** UNG: Uracil-DNA glycosylase 2. **B.** ß-POL: DNA polymerase beta. **C.** p21: Cyclin-dependent kinase inhibitor 1. **D.** p16: cyclin-dependent kinase inhibitor 2A. **E.** RAD21: Double-Strand-Break Repair Protein Rad21 Homolog. Data are presented as mean (± SEM), n=3. ANOVA $P<0.0001$. Values with different superscripts indicate significant differences at $p<0.05$.

Table 2.1: detailed description of lymphoblastoid cell lines (LCLs).

LCLs	Type	Tissue	Gender	Age	Ethnicity
GM00130	Control/normal	B-lymphocyte	male	25yr.	Caucasian
GM16113	Control/normal	B-lymphocyte	male	27 yr.	Caucasian
GM16118	Control/normal	B-lymphocyte	male	21 yr.	Caucasian

CHAPTER 3: THE RELATIONSHIP BETWEEN EXCESSIVE FOLIC ACID INTAKE AND GENOME INSTABILITY IN HUMAN LYMPHOCYTES OF HEALTHY ADULTS, FUNCTIONAL FOLATE DEFICIENCY

3.1 Abstract

Mandatory fortification of grain and grain products with synthetic folic acid (FA) was initiated in the U.S. in 1998 in an attempt to reduce neural tube defects (NTD). Since the introduction of FA fortification, there has been a significant increase in serum and red blood cells (RBC) folate levels in the U.S. associated with a 19-31% reduction in NTD. However, despite the reduction in NTD, several studies have shown a significant increase in the onset of colon and rectal cancer over this period. Unlike the natural form of folate, FA as a provitamin is required to be activated in a two-step reduction by dihydrofolate reductase (DHFR), a very slow process in human tissues. Thus, most absorbed FA appears in the blood as unmetabolized FA (UMFA). Furthermore, several studies have implicated UMFA with reduced natural killer cells and cancer progression. In this study, we proposed that the chronic and excessive exposure to FA via fortified food induces functional folate deficiency, resulting in genomic instability. Herein, we evaluated the correlation between high intake of FA via fortified food and the level of genome damage/instability in lymphocytes of healthy adults by cytokinesis-block micronucleus (CBMN) assay. In our study, we found a significant correlation between the highest tertile of FA intake (>200μg DFE, Dietary folate equivalent) and increased micronuclei (MNi), nucleoplasmic bridge (NPB) and nuclear buds (NBUD) scores (r = 0.38, 0.39 and 0.3, respectively). Whereas we observed a strong inverse correlation between low FA to total folate intake ratio (FAR) < 0.35 and the aforementioned markers (r = -0.43, -0.61

and -0.38), we also observed a strong positive association between high FAR > 0.5 and the aforementioned markers (r = 0.69, 0.67 and 0.51), respectively. Our data suggest that excessive FA intake and high FAR were associated with elevated genomic instability, i.e., an increase in cytome biomarkers, imitating folate deficiency.

3.2 Introduction:

Folate is an essential vitamin present naturally in green vegetables, liver, legumes and in some fruits. It acts as a cofactor for several enzymes involved in DNA biosynthesis, repair and maintenance [59, 60]. Impaired folate metabolism or folate deficiency has been linked to NTD [61] and carcinogenesis in a wide range of tissues, including breast, cervix and colon [40, 62, 63]. Several countries, including the U.S., Canada and Chile, have mandated fortification programs of grain and grain products with folic acid FA, a synthetic version of folate, in order to mitigate NTD [12, 16]. Even though there has been a successful reduction in NTD (50% in Chile, 46% in Canada and 19-31% in USA) [16], the number of cancer cases has increased steadily. For instance, colon cancer, which is widely studied and linked to folate deficiency, has increased rapidly in the U.S. after the mandatory fortification implementation, especially in younger generations[64], as well as in Chile [21]. Several researchers have pointed out that DNA methylation abnormalities are mostly the candidates explaining the link between folate and acrolectal cancer [4, 65, 66]. Folate (5-mTHF) is vital for the constant conversion of methionine to S-adenosylmethionine (SAM), a methyl group donor for cellular and DNA methylation pathways. Folate exists naturally as 5-mTHF or 5-formyl-THF, which can also be converted rapidly and efficiently by human intestinal mucosa to 5-mTHF. Unlike folate,

FA must be activated and converted to 5-mTHF mainly by DHFR and methylenetetrahydrofolate reductase (MTHFR) in multiple steps reactions [6, 36, 67]. This process of activation is very slow in humans, as illustrated by Patanwala et al. and Bailey et al., which makes most of absorbed FA appear in the blood as unmetabolized FA (UMFA), and this form has been found to persist in the blood even after 12 hours of fasting indicating poor handling by human tissues for FA [1, 3]. Once inside the cell, UMFA is capable of acting as a competitive and uncompetitive inhibitor for DHFR, depending on DHF concentration inside the cells [3]. DHF is generated naturally inside the cells during thymidylate synthesis, and it was found that DHFR coexists and shares transcription factors with thymidylate synthase (TS) [68, 69]. Therefore, the continuous inhibition of DHFR by FA leads to the accumulation of DHF molecules, which was shown to be a potent inhibitor of MTHFR in pig liver as demonstrated by Matthews et al. [70]. Further, the U-shaped association of FA intake has been implicated in several deleterious outcomes, including colon cancer [4, 71]. Accordingly, we proposed that the chronic and excessive exposure of FA through fortified food induces functional folate deficiency and consequent genomic instability at the cellular level of human lymphocytes. In addition, the severity of this impact depends greatly or partially on the amount of natural folate consumed with respect to genetic variability in folate absorption and metabolism-related enzymes and their relative cofactors, such as B12, B2, B3 and B6.

Lymphocytes (Lymph) are widely accepted as a suitable cellular source to examine the effect of folate status on genome instability markers, such as strand breakage, microsatellite instability, hypomethylation, and uracil misincorporation[72]. The CBMN

assay developed by Fenech et al. provides a comprehensive and well-validated method to measure endpoints of DNA damage, such as: MNi, biomarkers of chromosome breakage and/ or chromosome loss; NPB, biomarkers of DNA misrepair and/or telomere end effusion; NBUD, biomarkers of the elimination of amplified DNA and/or DNA repair complexes [2, 48, 49]. MNi is a very sensitive measure of small changes in micronutrient status, including folate, which makes it a robust biomarker to identify the impact of excessive folate intake on genome stability.

Basten et al. showed that Lymph total folate is highly sensitive to folate intake and correlates with plasma 5-m-THF and homocysteine, but not with RBC folate which indicate that lymph would be a better indicator to current changes in food intake [72]. However, to our knowledge, there is no available data about the lymph folate correlation level after the intervention of synthetic FA. A study by Kim et al. showed that colon folate correlates strongly with RBC folate and serum folate, but after the intervention with FA this correlation level dropped significantly after 6 months and disappeared completely after one year [73]. If this would be true for lymph tissues as well, it would mean neither of the conventional markers would accurately predict the folate status of lymph after the mandatory fortification. Hence, we need to find other markers that would predict folate status accurately. Biomarkers of Nutrition for Development (BOND) suggested in their folate review that MNi, uracil misincorporation and LINE-1 hypomethylation would serve as good surrogate markers for folate deficiency status [6]. Therefore, in this study, we evaluated the correlation level of excessive FA intake through

consumption of fortified food on genome instability of human lymphocytes using CBMN assay.

3.3 Materials and Methods

Recruitment and Data collection

A total of 57 induvials were recruited for this study. Participants were screened using our food folate survey to estimate individual food habit and folic acid intake. This study aimed to have 3 groups of population with 3 levels of folate intake as shown in **Fig S3.1**. Our inclusion criteria were healthy adult between the ages of 18 and 40 years old. Those undergoing a medical treatment for current disease, taking medications or drugs, pregnant and lactating women, strict vegetarians, vegans, heavy alcoholics, heavy smokers and B-vitamins or multivitamins consumers were excluded from this study. Thirty-three individuals were included in the study. At the first visit, participants read and signed informed consent, food diary instructions which were administered by trained personnel. We asked participants to record food intake for two days (one weekday and one weekend day) in the first week. Only those whose food intake analysis still met the estimated intake were asked to provide two extra days of food intake for the following week and donate blood samples. Participants anthropometric measurements were obtained as well. This study was approved by Institutional Review Board (IRB), Wayne State University, Detroit, MI.

Dietary Intake Measurements

Dietary intake was assessed using a 4-day food diary over two weeks (2 weekdays and 2 weekend days). Participants were asked to indicate details about the food item,

name, type, size, amount, labeling, recipe for home-cooked item, or the name of the restaurant. The food diaries were reviewed with participants by trained personnel to assure the portions and information of food were provided. Participants were contacted whenever any missing critical information was needed about their intake.

Food Intake, Folic acid intake Analysis

Food intakes were analyzed using *eSha* food processor nutrition analysis software [*74*] to attain macronutrient(protein), micronutrient (Iron, choline, B1, B2, B3, B6, B12, Folate) intake. However, since the software does not discriminate between natural folate and FA, we obtained the amount of FA from fortified food item by referring to Food Data Central on the USDA website [*75*]. Only the items with the amount of total folate (µg and µg DFE) that matched the *eSha* analysis software were considered.

Blood Samples Collection and Analysis

Participants donated their blood samples between 9:00 and 11:00 am after an overnight fast and before having breakfast to avoid possible effects of variation by dietary metabolites. Participants were also encouraged to drink water in the morning to avoid misleading complete blood count (CBC) results. Blood samples were collected by a certified phlebotomist; the samples were analyzed promptly either in our laboratory or in certified medical laboratories [*76*]. The lab analyses performed were serum folate, RBC folate, plasmatic homocysteine, serum B12, methylmalonic acid (MMA), plasmatic B2, B6, and CBC.

Cytokinesis-block micronucleus (CBMN) Assay

Blood samples were collected in K_2-EDTA tube and processed within two hours of collection. Lymphocytes were isolated using a density gradient medium (Lymphoprep ™, Stem Cell technologies, Germany). The CMBN assay in lymphocytes was performed using the protocol of Thomas and Fenech (2011)[42]. Briefly, the isolated lymphocytes were washed twice in a Hank balanced salt solution (HBSS), and then resuspended in culture media. Cell concentration was estimated using an automated cell counter. Cells were cultured at concentration of $1X10^6$ cells/ml in RPMI 1640 medium with 10% FBS, 1% penicillin, 1% glutamax and 300nM FA). All cultures were prepared in duplicate. Forty- four hours after phytohaemaglutinin (PHA) stimulation (45µg/ml), cytochalasin-B (4.5 mg/ml) was added, and cells were harvested 28 hours later on slides using Cytospin 4 (Shandon). Two slides were prepared for each duplicate culture, air dried, fixed in absolute methanol and stained using a Diff-Quick staining kit (Thermo Scientific). The frequency of MNi, NPB and NBUD was determined in 2000 binucleated (BN) cells following the scoring criteria of HUMN project guidelines [43]. Slides were coded and scored by two trained scorers who had no access to the codes. Scores presented as per 1000 BN cells, appendix **B**.

LINE-1 Methylation Assay

Genomic DNAs were isolated within 2 hours of collection in K_2-EDTA tube using a PureLink® Genomic DNA Mini Kit (life technologies, Carlsbad, CA), following the manufacture's protocols. LINE-1 methylation assay was performed using a Global DNA Methylation LINE-1 Kit (Active Motif, Carlsbad, CA).

Gene Expression Analysis

After blood collection in a K_2-EDTA tube, RNA *later* was promptly added to blood samples in an attempt to protect mRNA from fast degradation. Total RNA population was extracted from blood within 4 hours of collection using a RiboPure™ -Blood kit (Ambion). DNase I digestion (8 U/µL) was performed to remove contaminating genomic DNA from eluted RNA. The isolated mRNA was reverse transcribed using and following the protocols of ImProm-II™ Reverse Transcription System. Various gene expression levels were quantified using a real time quantitative polymerase chain reaction (qPCR), PikoReal 96 (Thermofisher, Vantaa, Finland). The Cq values were normalized to the geometric mean of HPRT1 and ß-Actin, and the gene expression was calculated using the equation – expression level of gene X = $2^{-(\Delta\Delta Cq)}$.

Statistical Analysis

Data are presented as mean (standard deviation). Mean comparison between two groups was performed using t-test. *Ad hoc* model analyses were performed on log transformed data using principle component analysis (PCA). One-way ANOVA was used to compare the means of 3 groups with *post hoc* t-test analysis. Pearson correlation coefficient was used to evaluate the correlation level between two variables. The receiver operating characteristic (ROC) curve was used to determine the sensitivity, specificity and area under the curve (AUC) to evaluate surrogate marker prediction levels. *P*-value < 0.05 was considered statistically significant. All data were statistically analyzed using the software programs SPSS 25.0 (IBM, Armonk, NY).

3.4 Results

General Participants Characteristics

The demographic and anthropometric measurement characteristics of the subjects in the study are listed in **Table 3.1**. The study was performed on 33 healthy individual (mean age 30.8 years, 36.4% females). There were statistically significant differences between males and females only in respect to body mass index (BMI) and waist to hip ratio (WHR). While females had lower values, males tended to have higher values. However, WHR values indicated most males were within the low-health risk ratio of developing cardiovascular diseases. Also, there were significant differences between genders in RBC count, hemoglobin (HGB), hematocrit (HCT) and RBC folate (supplementary data). Females had higher mean RBC folate (708 µg/L) than males (551 µg/L). RBC folate correlated inversely with RBC count, HGB and HCT (r= -0.5, p< 0.0001).

The relationship between cytome biomarker frequency scores and individual characteristics analysis indicated no significant association with MNi. However, BMI correlated positively with NPB (r= 0.35, p<0.04) and NBUD (r= 0.3, p< 0.08).

Systemic Markers Analysis

The evaluation of blood markers revealed that mean serum folate (SF) and B12, plasma B2 and B6 fell in the reference range. The absence of B12 deficiency was further confirmed by normal MMA value despite the observation of elevated plasma homocysteine (HC) level. Of the study population, 39% had moderate HC level (15-20 µmol/L), 33% high HC level (> 20 µmol/L), and 18% had high serum folate (> 20 µg/L).

The prevalence of RBC folate above the reference range (> 628 µg/L) occurred in 33% of participants. We also evaluated RBC indices for the presence of anemia and/or iron deficiency. Mean RBC count, HGB and HCT were within the normal range. Other RBC indices showed 33% macrocytosis (MCV > 100 fL) and 64 % hypochromia (MCHC < 32 g/dL), and 48% had high RDW-SD (> 46 fL), conditions that could be associated with folate and B12 deficiencies.

Individuals Nutrient Intake Analysis

The mean intake of protein, choline, iron, B1, B2, B3, B6, B12, folate is shown in **Table S3.1** with RDA and AI reference. We evaluated the individual intake of the aforementioned nutrients using recommended dietary intake values as they are fixed for gender, age and population. The analysis of **Fig 3.1** showed that aside from the variability of folate intakes, most participants met 100 % RDI. Slightly lower intakes of choline and B2 are indicated by the error bars. Conversely, protein and B12 intakes exceeded 150 % RDI.

Models Analysis

The analysis of PCA for systemic markers indicated that RBC folate had a minimal effect on the variation of the study population. Most of the variation was driven by SF, HC, MCV, RDW, MCHC and B2 for first principle component (PC1), and Lymph, Neut, WBC, MPV and B6 for PC2 as shown in **Fig S3.5 and Fig S3.8**. In addition, SF, HC, MCV, RDW, MCHC intercorrelated significantly and strongly with each other as expected, as shown in supplementary data. Since the relationships between folate status and SF, HC, MCV, MCHC, RDW have long been established previously, we proposed

4 *ad hoc* multivariate models using those markers to look into the relationship between folate intake, FA intake and the ratio between folic acid and folate intake. The models were generated based on the tertiles of serum folate (Model 1), total folate intake (Model 2), FA intake (Model 3) and FA to folate ratio (Model 4). The purpose of these models is to compare cytome biomarker scores between each tertile and look into the correlation level between each variable. The detailed descriptions of the models and PCA analysis of the models are presented in supplementary data. The analysis of Model 3, based on FA intake tertiles (**Fig S3.3**), revealed that a mean MNi score of the highest tertile was statistically significant and higher than the lowest tertile. The highest tertile of folic acid intake (>200 µg) correlated positively and significantly with MNi, NPB and NBUD (r= 0.39, 0.39, 0.3 p<0.05)), respectively. The lowest tertile of FA intake (< 100 µg) correlated inversely and significantly with MNi (r= -0.39 p<0.01).

Model 4, based on FA ratio, analysis revealed a clear separation of all cytome biomarkers between the third tertile and the first and second tertiles. Mean MNi, NPB and NBUD differed significantly between the third tertile and first and second tertiles (**Fig 3.2**) but were not statistically significant between the first and second tertiles. Whereas the highest tertile of folic acid ratio (> 0.5) correlated positively and significantly with MNi, NPB and NBUD (r= 0.69, 0.67 and 0.51, P<0.001) respectively, the lowest tertile of FA ratio (< 0.3) correlated significantly and inversely with the aforementioned cytome biomarkers (r= -0.43, -0.61 and -0.38 p< 0.01), respectively.

There were neither significant association between cytome biomarkers and other models' variables, nor significant results obtained from Model 1, based on serum folate, and Model 2, based on total folate intake as well (**Fig S3.2 & Fig S3.3**).

LINE-1 methylation level of Model 4:

We proposed that the functional folate deficiency induced impaired methylation associated with increment in genome damage. We utilized the LINE-1 methylation level as a surrogate marker for global genome methylation. We found a reduction in mean methylation level in the third tertile though it was not significant, $p = 0.07$, **Fig 3.3**.

Gene expression Analyses:

We performed gene expression analyses for 6 genes in an attempt to compare the second and third tertiles of folic acid ratio because they were more homogeneous; no significant difference was observed between these tertiles in BMI level. As shown in **Fig S3.4**, we found a 0.5-fold increase in O-6-methylguanine-DNA methyltransferase (MGMT) gene expression level in the third tertile when compared with second and first tertiles. This increase was also associated with a 1.5-fold increase in mutL homolog 1(MLH1), and 1-fold decrease in uracil DNA glycosylase (UNG) and cyclin-dependent kinase inhibitor 2A, CDKN2A, known as p16. While MGMT, UNG, MLH1 are all genes that participates in various DNA repair machinery, p16 is a tumor suppressor gene. There also was 1.5-fold increment in the third tertile of both 5,10-methelenetetrahydrofolate-reducatase (MTHFR) and 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), which is also known as Methionine synthase (MS), a B12 dependent enzyme. Both MTHFR and MTR participate in folate

metabolism and folate-methylation flux. However, these results were not statistically significant.

Cytome biomarkers and specific nutrients intake Analysis

We looked into the association between cytome biomarkers and other B-vitamins, iron, protein and choline intake. PCA analysis in **Fig 3.4** indicated that there was an inverse association between MNi, NPB & NBUD and choline (r= -0.33, -0.30 and -0.45 p< 0.01), and protein intake as well (r= -0.28, -0.25 and -0.47 p< 0.05), respectively.

Surrogate Markers analyses

Serum folate values did not provide discrimination between 5-mTHF and UMFA and do not reflect current tissue status. In addition, since food diary intake method has its limitations, we searched for other markers that would serve as surrogate markers for folate status. Unlike RBC folate and RBC indices which reflect long term of tissues folate storage (the last ~120 days), white blood cells (WBC) have a shorter life span (days to weeks) [*77*]. We evaluated WBC count and WBC indices association in respect to cytome biomarkers. PCA loading plot **Fig S3.5** showed that MNi, NPB & NBUD correlated positively with Lymph percentage (%) (r= 0.58, 0.50 and -0.55 p< 0.01) and correlated inversely with Neutrophils percentage (Neut %) (r= -0.54, -0.63 and -0.53 p< 0.01), respectively. They similarly correlated inversely with Neut /Lymph ratio (NLR) (r= -0.43, -0.46 and -0.33 p< 0.001), respectively. Additionally, MNi correlated positively and significantly with mean palatal volume (MPV) (r= 0.38, p< 0.01) and MPV to palatal count (MPV/PC) (r= 0.3, p< 0.05), and NPB correlated negatively with WBC count (r= -

0.4, $p<$ 0.01). NPB and NBUD correlated positively with Monocytes percentage (Mono %) (r= -0.4 and -0.26 $p<$ 0.01).

Then, when we compared the fitting of the second and third tertiles of FAR to the aforementioned surrogate markers, we found that the third tertiles correlated significantly with WBC, Neut (%), Lymph (%), Mono (%), Palatal (PLT), MPV, MPV/PC and NLR as follow: r(p)= -0.4 (<0.01), -0.61(<0.001), 0.68(<0.001), 0.24 (<0.01), -0.32 (0.03), 0.34 (0.02), 0.38 (0.01) and -0.62(<0.001). The second tertile correlated significantly with WBC, Neut, Lymph, Mono, PLT, MPV, MPV/PC and NLR as follow: r(p)= 0.46 (<0.01), 0.60 (<0.001), -0.61 (<0.001),- 0.54 (<0.01), 0.36 (0.01), -0.43 (<0.001), -0.43 (<0.01) and 0.52(<0.001). The association between the surrogate markers and the third tertile of FAR was similar to the association between those markers and cytome biomarkers (**Fig 3.5)**, whereas the second tertile was mutually opposite the third tertile and cytome biomarkers.

Afterward, we compared the mean of each surrogate marker between the second and third tertiles. Our data (**Fig 3.6)** indicated significant differences in terms of WBC, Neut (%), Lymph (%), Mono (%) and NLR, but there were no significant differences in terms of PLT, MPV, MPV/PC (p= 0.07) and LMR (p=0.06).

Furthermore, we used the receiver operator characteristic ROC curve to discriminate among surrogate markers ability to predict the FAR (cut point > 0.50); cytome biomarkers (cut point > 20-MNi,10-NPB and 10-NBUD). MNi, NPB and NBUD intercorrelated strongly, r= 0.8, p< 0.0001 (data not shown). AUC provides a meaningful interpretation about the accuracy of each measure [78], presented as mean

with 95% confidence interval (CI). For FAR prediction **(Fig 3.7A)**, Lymph (%) had higher sensitivity, specificity, and AUC= 0.92 (0.8-1), p<0.001, 91% sensitivity and 76 % specificity. The MPV/PC ratio had lower sensitivity and specificity, but AUC= 0.72 (0.55-0.89), p=0.03, 75% sensitivity and 62 % specificity. For the prediction of cytome biomarkers **(Fig 3.7B)**, only Lymph (%) had a great AUC = 0.89 (0.78-1), p<0.001, with 82% sensitivity and 86 % specificity.

Finally, we compared the mean of first tertile and second tertile of FAR. Besides the significant difference in BMI mean (SD) 28.8 (4.8),and 23.5 (3.6), p<0.05, they significantly differ in Mono (%) 8.9 (1.7) and 6.4 (1.3), p<0.05, and in LMR 3.98 (0.8) and 4.86 (1.4), p<0.05, respectively. The first tertile of FAR correspondingly correlated significantly with Mono (r= 0.28 p= 0.04) and with LMR (r= -0.38 p= 0.01). BMI also correlated to similar degree with Mono (r= 0.29 p= 0.04), and the Mono AUC analysis for BMI > 30 kg/m^2 was 0.75 (0.58-0.92) p=0.02 **(Fig 3.7C)** with 80 % sensitivity and 53% specificity.

3.5 Discussion

The association between folate deficiency and cytome biomarkers in human lymphocytes has long been established, as well as adequate folate status as a precursor for genomic stability has also long been determined. Several studies evaluated the impact of FA deficiency/adequacy on MNi, NPB and NBUD formation in human lymphocytes *in vitro* [8, 25, 79, 80]. Thus, the assumption of the benefit of high folate intake in the diet was indomitable. However, studies examined this association *in vivo*, by evaluating either serum folate, RBC folate, homocysteine level or folate intake, have found conflicting

results. Also, we are not aware of any published studies that have examined the impact of excessive FA intake on MNi, NPB and NBUD formation in human lymphocytes *in vitro* or *in vivo*. Besides, most food intake studies evaluated total folate intake, or FA intake without elucidating the amount of natural folate and the synthetic form. Therefore, the primary aim of this study was to determine whether excessive intake of FA through the consumption of fortified food is associated with genome instability in human lymphocytes of healthy adults.

We proposed that excessive FA intake would induce functional folate deficiency and consequential DNA damage. We evaluated the relationship between serum folate, total folate intake, FA intake, FAR to total folate intake and DNA damage as indicated by the presence of cytome biomarkers. Our analyses indicated that neither serum folate nor RBC folate correlated with cytome biomarkers which corroborates the finding of Fenech et al [8, 26, 81]. The analysis of mean cytome biomarkers between serum folate tertiles and total folate intake tertiles revealed no significant differences; those findings are similar to the findings of Fenech et al.[81] but also disagree with the results of other studies [82, 83]. The disagreement can be explained by the approach of each study. Both studies compared MNi to the baseline where the folate intake is below the recommended level, i.e., high MNi is related to folate deficiency. Serum folate is known to correlate strongly with folate intake, and both parameters do not distinguish between natural form of folate or the synthetic one [34, 72]. A study by Ladeira et al. used food a frequency questionnaire and found no significant correlation between total folate intake (mean 401 ± 24 μg) and MNi, NPB and NBUD [84]. Therefore, we compared the mean of cytome

biomarkers based on the tertiles of FA intake and FAR tertiles. Our results denoted significantly higher MNi, NPB and NBUD frequencies in the highest tertile of FA intake (>200 μg DFE) when compared with the lowest tertile (<100 μg DFE). To our knowledge, there is no published data that elucidates the effect of FA intake alone as food fortificant on genome damage in humans.

High MNi, NPB and NBUD frequencies were observed in the highest tertile of FAR (>0.5) when compared with second and first tertiles (<0.35). Also, FAR > 0.5 correlated strongly with cytome biomarkers with the lower ratio (< 0.35) appearing to provide protection from those biomarkers. The findings of the current study support our proposal that excessive FA intake induces a functional folate deficiency and this insufficiency is resolved by the interference of 5-mTHF (natural folate) species from natural sources. We are not aware of any published data that consider the ratio of FA to natural folate intake, yet a model that supports our data is Methotrexate (MTX), a chemotherapeutic drug used to treat some cancers and rheumatoid arthritis. MTX is a DHF analog that inhibits DHFR. Thus, treatment with low dose of MTX yields side effects that mimic folate deficiency[85]. In addition, the result of the study by Shahin et al. evidently indicated that MTX treatment significantly induced MNi frequency in bone marrow cells of MTX-treated rats as well as in peripheral blood cells of MTX-treated rheumatoid arthritis patients [86]. Madhyastha et al. also demonstrated that Leucovorin, folinic acid (5-formyl-THF), has a protective effect against MTX-provoked MNi formation in rat bone marrow, i.e. supplementation with Leucovorin following MTX treatment decrease the formation of MNi significantly [87]. Our data showed similar

observation that low FAR had a protective effect and was associated significantly with lower formation of MNi, NPB and NBUD as well. This observation can be explained that the lower the FAR, the higher the natural folate intake. Since the natural form, either 5-m-THF or 5-f-THF, does not require the action of DHFR to be activated [60], its function is unaffected by the inhibition of DHFR by excess FA intake. Therefore, natural folate allows the synthesis of purine/pyrimidine even in the absence of DHFR activity, and in turn, normal DNA replication/repair and RNA transcription processes can proceed[87]. Additionally, 5-m-THF surpasses FA in terms of feeding the cellular methylation pathway. It donates a methyl group once entering the cell in B12-dependent reaction to convert homocysteine to methionine in SAH to SAM pathway without reliance on DHFR activity [36]. Hence, less genome damage was induced, i.e., less cytome biomarkers, were observed in the first and second tertiles of FAR.

Homocysteine and B12 were found previously to correlate positively and inversely with MNi scores in lymphocytes of male adults, respectively [26, 81]. In general, we did not see in general a similar significant association pattern in our samples. This could be due to the small size of our sample and/or due to the relatively variable and high mean homocysteine level despite the normal values of serum folate, B12 and MMA. Little is known about the effect of chronic exposure of FA in the diet on homocysteine level. This difference could be explained that the reduction found in MNi in those studies was attributed to the high rate of chromosomal damage at the baseline. Besides, those studies were before the era of FA fortification, and there is little known about the impact of long-term FA supplementation on HC level, as is the case with mandatory fortification.

However, HC level reached a plateau and increased again when RBC folate level exceeded the reference range in elderly populations with Alzheimer disease [88]. This could explain the high and wide variation in homocysteine level in our samples.

The relationship between HC, B12, FAR could indicate that the underlying mechanism of MNi formation was attributed to hypomethylation events rather than DNA breakage. We found no significant differences between UNG gene expression between FAR tertiles, yet the third tertile was the lowest. CpG hypomethylation was shown to be associated with MNi formation in healthy young males [26]. Hence, we evaluated the methylation status using LINE-1 methylation as a surrogate marker of global genome methylation [6]. LINE-1 hypomethylation was reflected with increasing MNi frequency in human lymphocytes [89]. Charles et al. showed that a supraphysiological level of FA induces LINE-1 hypomethylation in a tissue and passage dependent manner [45]. We found no significant difference in LINE-1 methylation between FAR tertiles ($p=0.07$) although a trend was observed, whereas the third tertile had the lowest methylation level.

Then, an analysis of nutrients intakes that have an impact on the activity of several enzymes that regulate folate metabolism, such as B12 (MTR), B2 (MTHFR) and B6 (SHMT)[67]. According to the PCA analysis, only protein and choline intakes correlated significantly and inversely with cytome biomarkers, which further supports our hypothesis. Protein is essential for methionine intake, as choline is for the production of betaine which remethylates homocysteine independently from B12 pathways[90, 91], and therefore increases methylation reactions.

MNi was proposed as a robust surrogate marker for folate status, but it is not specific for folate unless combined with uracil misincorporation [6]. Unfortunately, at this point we could not perform the analysis for uracil misincorporation, nor identify the dNTPs pools ratio. Therefore, the secondary objective of the current study was to identify conventional surrogate markers for folate status insufficiency that could be linked to cytome biomarkers. Several studies have linked UMFA to several adverse health outcomes in human, such as reduced natural killer (NK) cytotoxicity [18]. It has been observed that either excessive or deficient folate status is related to alteration in the immune response as illustrated by Henry et al. [55]. FA also is known to induce lymphocytes generation in the bone marrow, and folate deficiency (FD) decreases WBC count, neutrophils, and the subset of lymphocytes, where B-lymphocytes and neutrophils are more sensitive to FD [92]. Moreover, FD has a direct impact on thrombocytes by decreasing the palatal (PLT) count while increasing their mean volume [93, 94]. These markers could be obtained as a component of CBC. Hence, we evaluated the relationship between cytome biomarkers and some proposed surrogate markers. Then we compared the mean of these surrogate markers between the second and third tertiles of FAR. We found that Lymph (%), Mono (%), NLR and MPV/PC were higher in the third tertile of FAR, while WBC, Neut (%) were lower in the third tertile. By examining the AUC of each surrogate marker, only Lymph (%) was significant when we set the cutoff point at > (20 MNi 10 NBP & NBUD). MPV/PC and Lymph (%) were significant when the cutoff point set at > 0.5 FAR.

The increase in Lymph (%) is associated with a simultaneous decrease in Neut (%) which indicates a similar underlying mechanism. Lymph (%) (i.e., the ratio of Lymph to WBC), which is considered to be a more accurate measure for immunity status than Lymph count alone [95]. It is also affected by Neut and Mono counts which accurately reflect the status of systemic anti-inflammatory surveillance, which inhibits tumor cell proliferation. Low Lymph (%) was found to be associated with lung cancer progression and as an independent prognostic factor in poor cancer treatment outcome[96].

Butin-Israeli et al. reported that Neutrophils can also encounter MNi formation as a result of genome damage[97]. Rello-veronal et al. showed that MNi formed in the cytoplasm can be sequestered and cleared by macroautophagy [98]. MNi was found to induce innate immune response to be cleared through autophagy via cGAS-STING pathway [99], DNA sensing machinery principal in the innate immune response [100]. Further research is needed to find which impact FD would have on this pathway as well as which kind of interplay between mechanistic target of rapamycin (mTOR) pathways for folate sensing, consequential autophagy and DNA sensing exists. Nonetheless, autophagy has been found to play a vital role in the development and differentiation of leukocytes and an essential role in lymph homeostasis by involving in the maintenance of certain lymph subtypes [101]. Unlike other WBC species, autophagy has a negative impact on Neut development and granulopoiesis[102, 103]. This would explain the simultaneous decrease in Neut (%) with the increase in Lymph (%) in our data. The decline in neutrophil count is common after chemotherapy, including MTX, albeit

neutropenia was found to be exacerbated in patients with high serum folate level (>20 ng/ml).

MNi also can be caused by increased uracil misincorporation into DNA, strand breakage or base lesions in DNA due to oxidative stress and alkylation [49]. MGMT gene participates in the removal of O-6-methyl guanine in DNA as a result of alkylating agents, one of which is S-adenosyl-methionine (SAM) as an endogenous source [104]. Defects in DNA repair machinery and inappropriate expression of genes associated with cell cycle checkpoints, such as p16, can induce MNi formation as well [24, 105]. Unfortunately, we did not find any significant differences (supplementary data). This could be a result of our method, i.e., isolating total transcript from whole blood. Therefore, sub fractioning of the lymphocytes approach with stabilizing mRNA integrity is required.

Our data support the finding of Fenech et al. that BMI has no effect on MNi scores [82]. However, we found that BMI has additive effects to NPB and NBUD scores as appeared from the comparison between the first and second tertiles of FAR. Mono (%) increased as a response of chronic inflammation or neutropenia. Chronic inflammation, i.e., increases in leukocytes and monocytes numbers, is associated with obesity and increased BMI as well [106, 107]. The significant intercorrelations between NPB, NBUD, BMI and Mono (%) indicate an underlying mechanism that could be related to chronic inflammation and oxidative stress. The high value of AUC for Mono (%) (**Fig.S3)** suggests that Mono (%) could be a valid screening tool with BMI for NPB and NBUD.

In conclusion, our data indicated strong correlations between excessive and chronic exposure to folic acid via fortified food and genome instability as well as an

alteration in the immunity response, and this relationship resembled functional folate deficiency. However, we are aware of the inherent limitation of food intake assessment instruments, such as food intake diary. Thus, given the small size of our sample in the current study, our results require further confirmation with a larger group of participants. This is a prospective observational study, and our findings will help to concrete more controlled clinical studies in the future.

Table 3.1: Description of main characteristics of study subjects.

Characteristic	N	Mean (SD)		Reference range:
Age (years)	33	30.80 (4.89)		
Males (%)	21	63.6 %		
BMI (kg/m^2)	33	26.14 (5.35)		Normal weight: 18.5-25
	M (21)	27.9 (5.5)		Overweight: 25-30
	F (12)	23.7 (4.2)	p = 0.03	Obese: >30
WHR	33	0.85 (0.08)		Low health risk:
	M (21)	0.88 (0.07)		M < 0.9
	F (12)	0.78 (0.06)	p< 0.001	F < 0.85

N, number of participants; **SD**, standard deviation; **M**, males; **F**, Females. **BMI**: body mass index. **WHR**: waist to hip ratio. p-value refers to comparison between M &F.

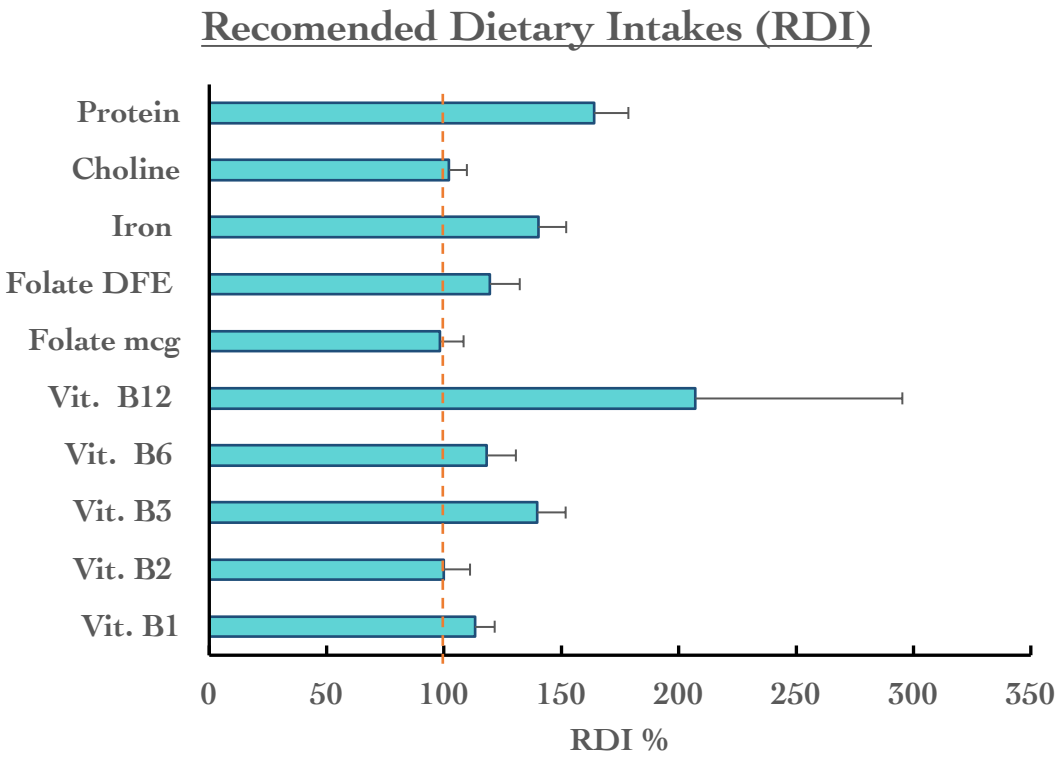


Figure 3.1: Average recommended dietary intake of study participants. Food intake were obtained by food diary intake for 4 days and analyzed by e*S*ha food processor. Data were presented as mean (± SEM), n=33. Vit. B1: Thiamine intake (mg), Vit.B2: Riboflavin intake (mg), Vit.B3: Niacin intake (mg), Vit.B6: Pyridoxin intake (mg), Vit.B12: Cobalamin (mcg), Folate. DFE: total folate intake (mcg DFE), Folate.mcg: total folate intake (mcg) Protein: protein intake (g), Choline: Choline intake (mg), Iron: Iron intake (mg). DFE, dietary folate equivalent.

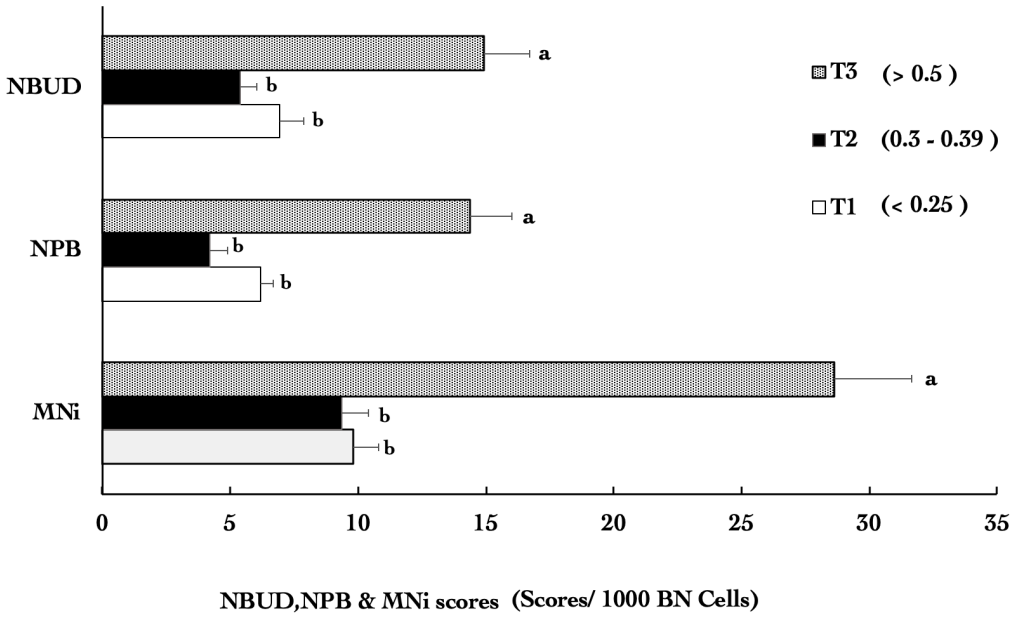


Figure 3.2: Cytome Biomarker frequency in human lymphocytes based on FAR tertiles (Model 4). MNi, NPB & NBUD scores were measured by CBMN assay, scores represented per 1000 binucleated (BN) cells. LCL. MNi: micronuclei, NPB: nucleoplasmic bridge, NBUD: nuclear bud. Lymphocytes were isolated from human participants divided into tertiles (T1, T2, T3) based on folic acid intake to total folate ratio (FAR). FAR were obtained by dividing folic acid intake on total folate intake. Data were presented as mean (± SEM). Values with different superscripts indicate significant differences at $p<0.05$. ANOVA $P<0.001$.

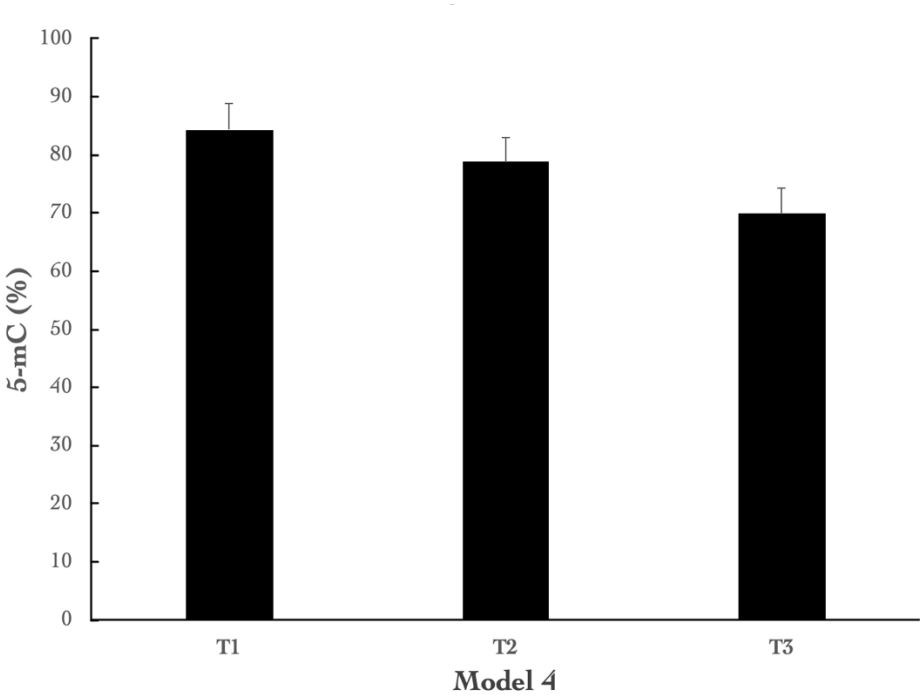


Figure 3.3: Global LINE-1 methylation level with respect to model 4, FAR tertiles. Level of 5-mC % was associated with detectable CpG residues in human genomic DNA by LINE-1 methylation assay. Genomic DNA was isolated from blood samples of study participants. Data were presented as mean (± SEM), n=33. ANOVA= 0.07.

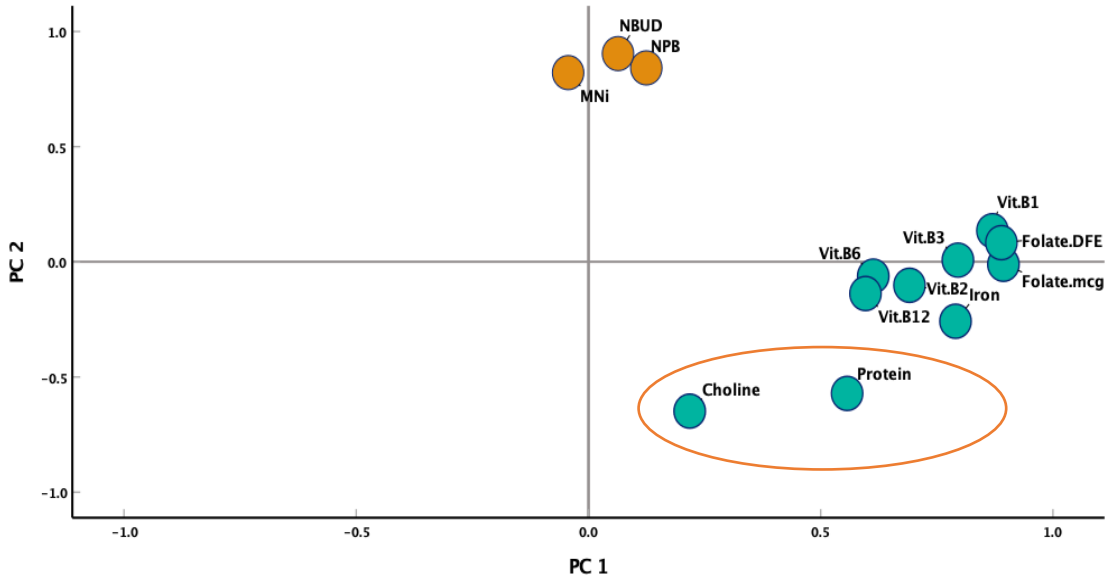


Figure 3.4: PCA loading plot of the correlation between cytome biomarkers & specific nutrients intakes. Food intake was obtained by food diary intake for 4 days and analyzed by e*S*ha food processor. MNi, NPB & NBUD scores were measured by CBMN assay. Data were presented as mean (± SEM), n=33. PCA cumulative variance explained (63.55%); PC1 (40.91%), PC2(22.64%), KMO=0.72, p-value < 0.0001. MNi: micronuclei, NPB: nucleoplasmic bridges, NBUD: nuclear bud. Vit. B1: Thiamine intake (mg), Vit.B2: Riboflavin intake (mg), Vit.B3: Niacin intake (mg), Vit.B6: Pyridoxin intake (mg), Vit.B12: Cobalamin (mcg), Folate. DFE: total folate intake (mcg DFE), Folate.mcg: total folate intake (mcg) Protein: protein intake (g), Choline: Choline intake (mg), Iron: Iron intake (mg).

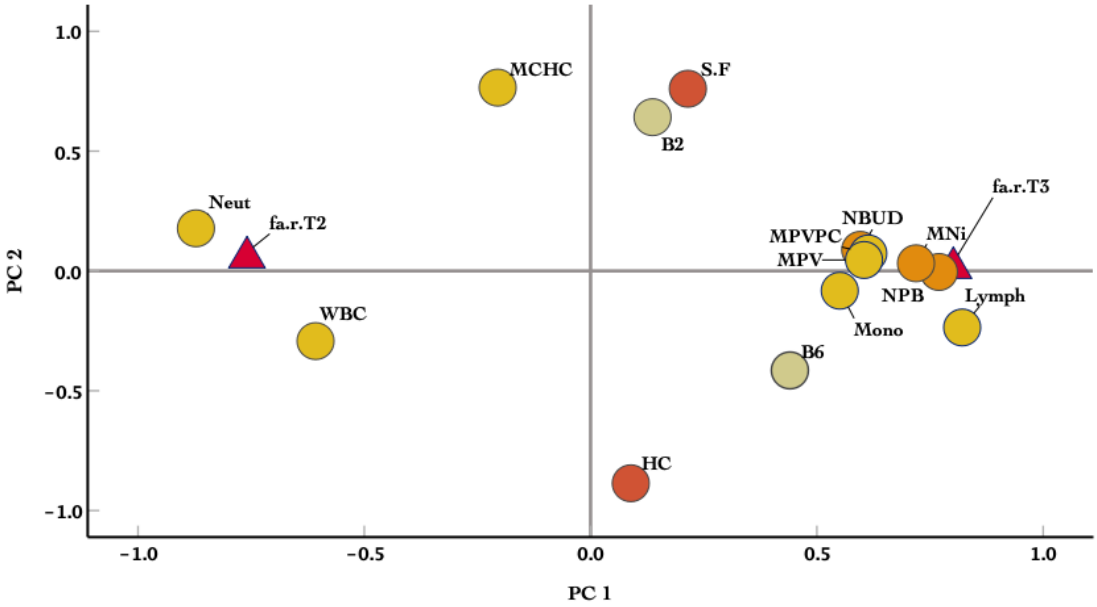


Figure 3.5: PCA loading plot of possible surrogate markers and their relationship with cytome biomarkers and FAR tertiles. PCA cumulative variance explained (53.6%); PC1 (36.7%), PC2(16.9%), KMO=0.69, p-value < 0.0001. fa.r.T2: second tertile of folic acid ratio, fa.rT3: highest tertile of folic acid to ratio. S.F: Serum Folate (µg/L), HC: plasma Homocysteine (µmol/L), B2: plasma Riboflavin (µg/L), B6: plasma Pyridoxal phosphate (µg/L), MCHC: Mean Corpuscular Hemoglobin Concentration (d/dL), MPV: Mean platelet volume (fL), MPV/PC: MPV/ Platelet count ratio. WBC: White blood cells (10^9/L), Neut: Neutrophils (%), Lymph: Lymphocytes (%), Mono: Monocytes (%). MNi: Micronuclei, NPB: nucleoplasmic bridges, NBUD: Nuclear bud.

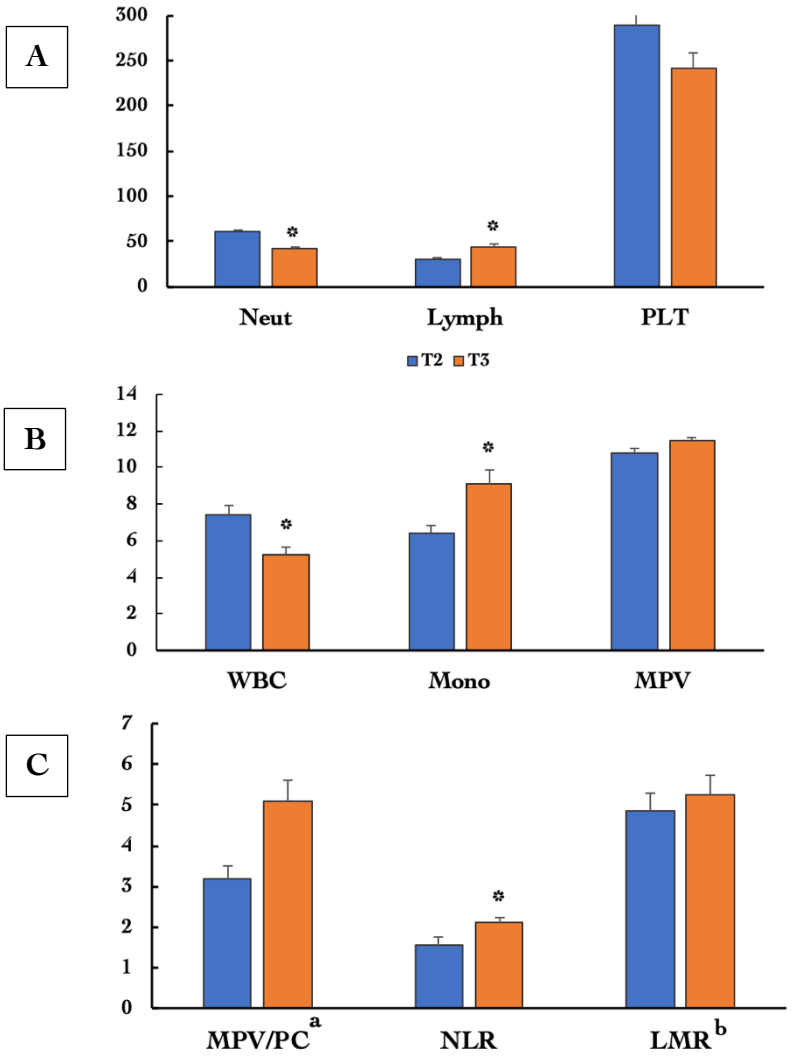
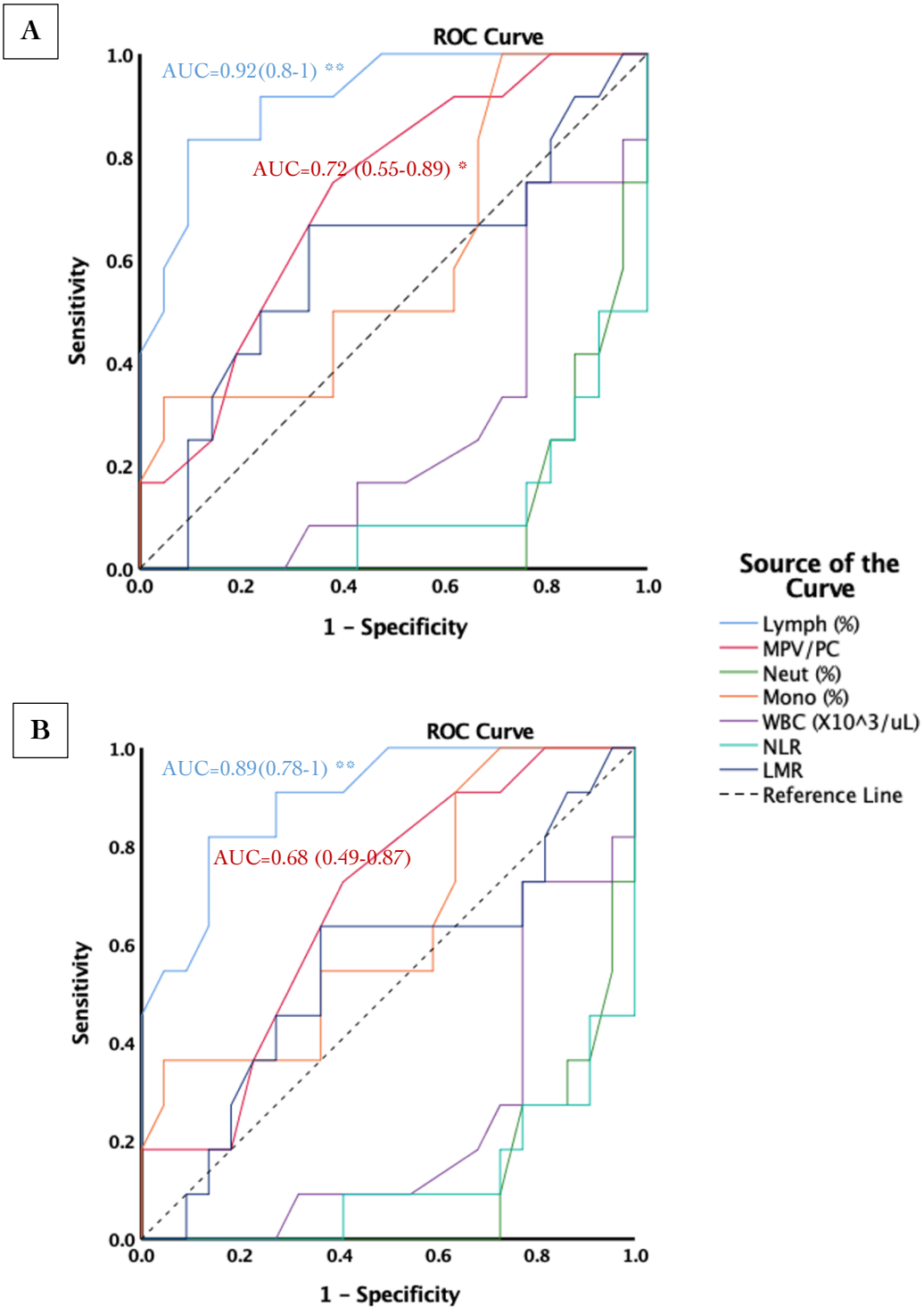


Figure 3.6: Comparison of possible surrogate markers between second and third tertile of FAR. Data were presented as mean (± SEM), n=22. A, Neut: Neutrophils (%), Lymph: Lymphocytes (%), PLT: Platelet count. B, WBC: White blood cells (10^9/L), Mono: Monocytes (%), MPV: Mean platelet volume (fL). C, a, values are multiplied with 100, p=0.06; b, p= 0.08. MPV/PC: MPV/PLT, NLR: Neut/Lymph, LMR: Lymph/Mono. T2: second tertile of FAR, T3: third tertile of FAR. * p< 0.01.


A
ROC Curve
AUC=0.92(0.8-1) **
AUC=0.72 (0.55-0.89) *
Sensitivity
1 – Specificity
0.0
0.2
0.4
0.6
0.8
1.0
B
ROC Curve
AUC=0.89(0.78-1) **
AUC=0.68 (0.49-0.87)
Sensitivity
1 – Specificity
Source of the Curve
Lymph (%)
MPV/PC
Neut (%)
Mono (%)
WBC (X10^3/uL)
NLR
LMR
Reference Line

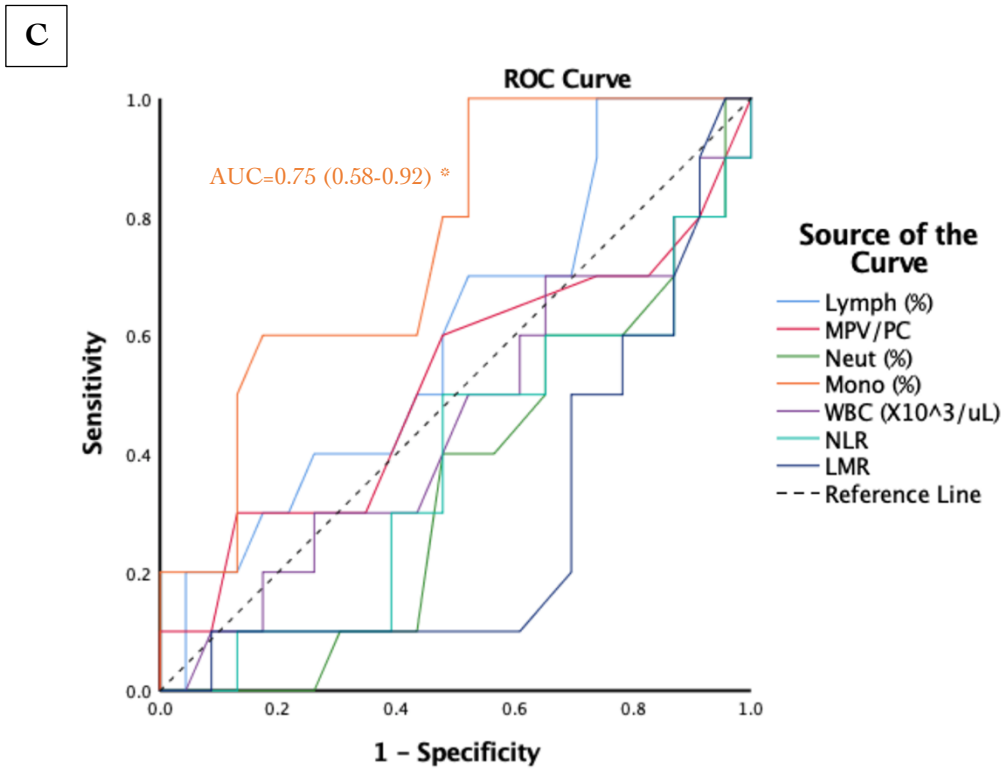


Figure 3.7: Comparison of prediction accuracy levels of possible surrogate markers. A, ROC curves of possible surrogate markers were in response to Folic acid ratio (FAR > 0.5)**. B,** ROC curves of possible surrogate markers were in response to cytome biomarkers (> 20-MNi, 10-NPBs &10-NBUDs). **C,** ROC curves of possible surrogate markers were in response to BMI > 30. Neut: Neutrophils (%), Lymph: Lymphocytes (%), WBC: White blood cells (10^3/µL), Mono: Monocytes (%), MPV/PC: MPV/PLT (MPV: Mean platelet volume (fL)/ PLT: Platelet count), NLR: Neut/Lymph, LMR: Lymph/Mono. Reference Line =0.5. AUC, area under the curve, mean (95% CI). * p< 0.05, ** p<0.001.

Table 3.2: Summary of significant correlations between cytome biomarkers and other variables.

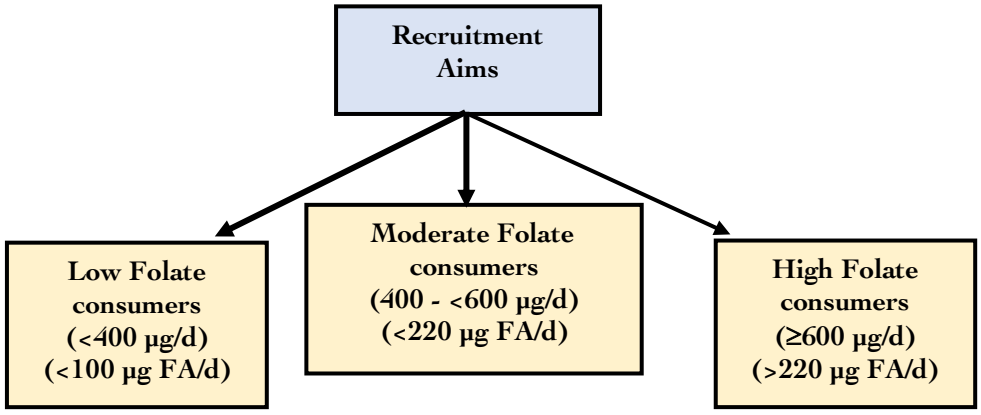
Pearson *r*	**MNi**	**NPB**	**NBUD**
Characteristics:			
BMI	ns	0.35 *	0.3 [a]
Nutrient Intake:			
Folic acid (µg)	0.39 *	0.41 **	0.42 **
Folic acid ratio	0.68 ***	0.51 ***	0.42 **
Protein (g)	-0.28 *	-0.25 *	-0.47 *
Choline (mg)	-0.33 **	-0.30 **	-0.45 **
Models Tertiles:			
fa.T1 (< 100 µg)	-0.39 **	ns	ns
fa.T3 (> 200 µg)	0.39 **	0.39 **	0.3 *
fa.r.T2 (< 0.3)	-0.43 **	-0.61 ***	-0.38 **
fa.r.T3 (> 0.5)	0.69 ***	0.67 ***	0.51 ***
Surrogate markers:			
WBC (10^3/µL)	-0.25 [a]	-0.45 ***	-0.34 *
Neut (%)	-0.54 ***	-0.63 ***	-0.53 ***
Lymph (%)	0.58 ***	0.50 ***	0.50 ***
Mono (%)	ns	0.32 *	0.32 [a]
MPV (fL)	0.37 *	ns	ns

p-value: * <0.05, ** <0.01, *** <0.001, a= ns, ns: not significant.

BMI: body mass index (kg/m^2), fa.T: folic acid intake tertile (Model 3), fa.r.T: folic acid intake ratio tertile (Model 4). WBC: White blood cells, Neut: Neutrophils, Lymph: Lymphocytes, Mono: Monocytes, MPV: Mean platelet volume. MNi: Micronuclei, NPB: nucleoplasmic bridges, NBUD: Nuclear bud.

Supplementary Figures:

Initial Recruitment → Screening (Using designed Folate - Food Intake Survey) *



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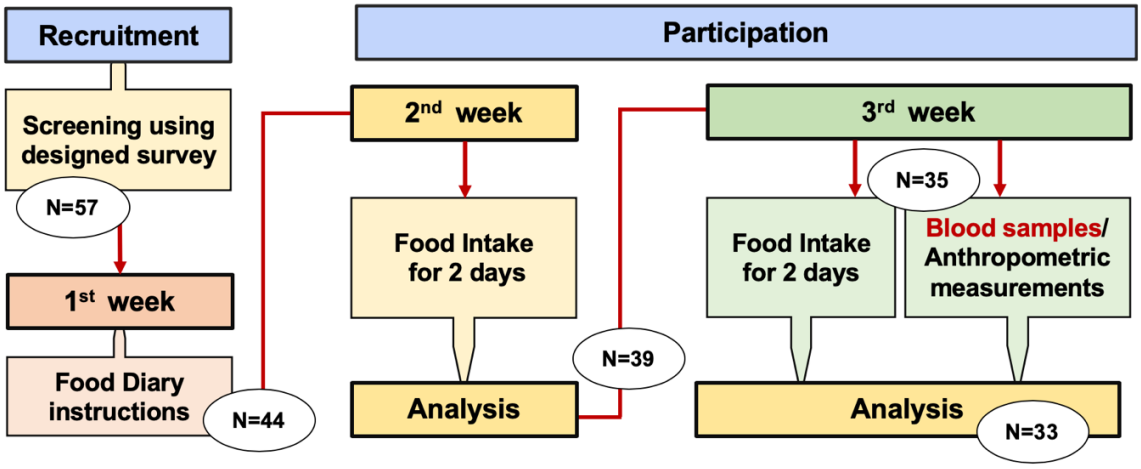


Figure S3.1: Study design depicting initial recruitment aims and data collection process. N= number of participants, * Appendix A.

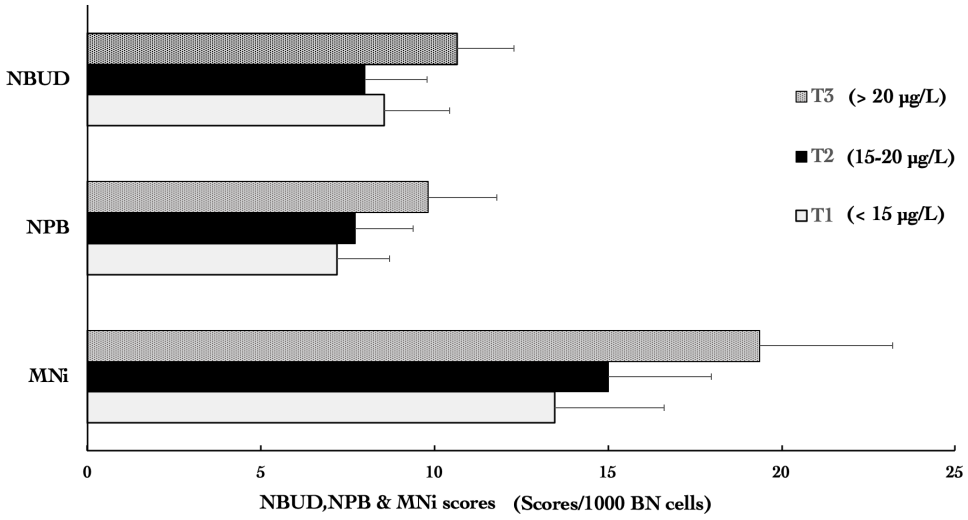


Figure S3.2: Cytome Biomarker frequency in human lymphocytes based on serum folate tertiles (Model 1). MNi, NPB & NBUD scores were measured by CBMN assay, scores represented per 1000 binucleated (BN) cells. LCL. Lymphocytes were isolated from human participants divided into tertiles (T1, T2, T3) based on the serum folate level. Serum folate presented as µg/L (ng/ml). Data were presented as mean (± SEM). ANOVA *P*.

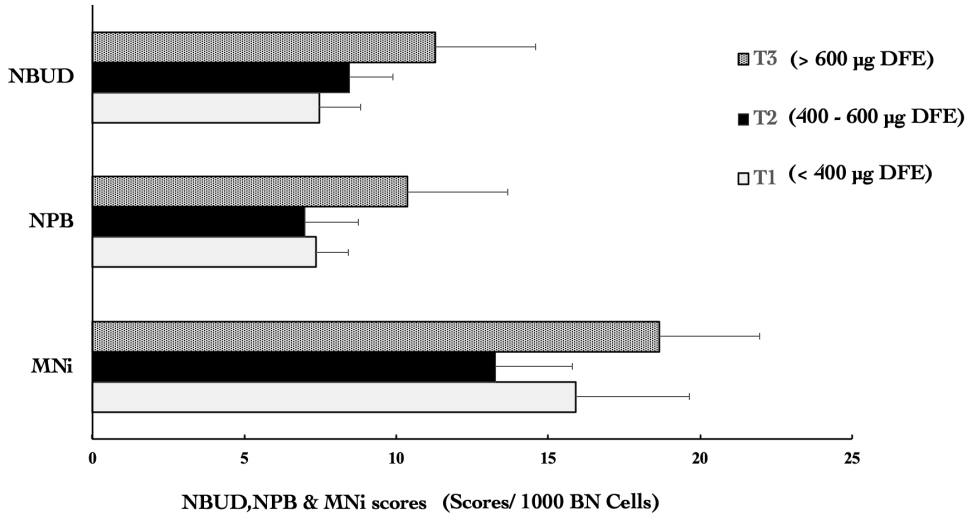


Figure S3.3: Cytome Biomarker frequency in human lymphocytes based on total folate intake tertiles (Model 2). MNi, NPB & NBUD scores were measured by CBMN assay, scores represented per 1000 binucleated (BN) cells. LCL. MNi: micronuclei, NPB: nucleoplasmic bridge, NBUD: nuclear bud. Lymphocytes were isolated from human participants divided into tertiles (T1, T2, T3) based on total folate intake. Total folate intake presented as µg DFE (dietary folate equivalent). Data were presented as mean (± SEM). ANOVA.

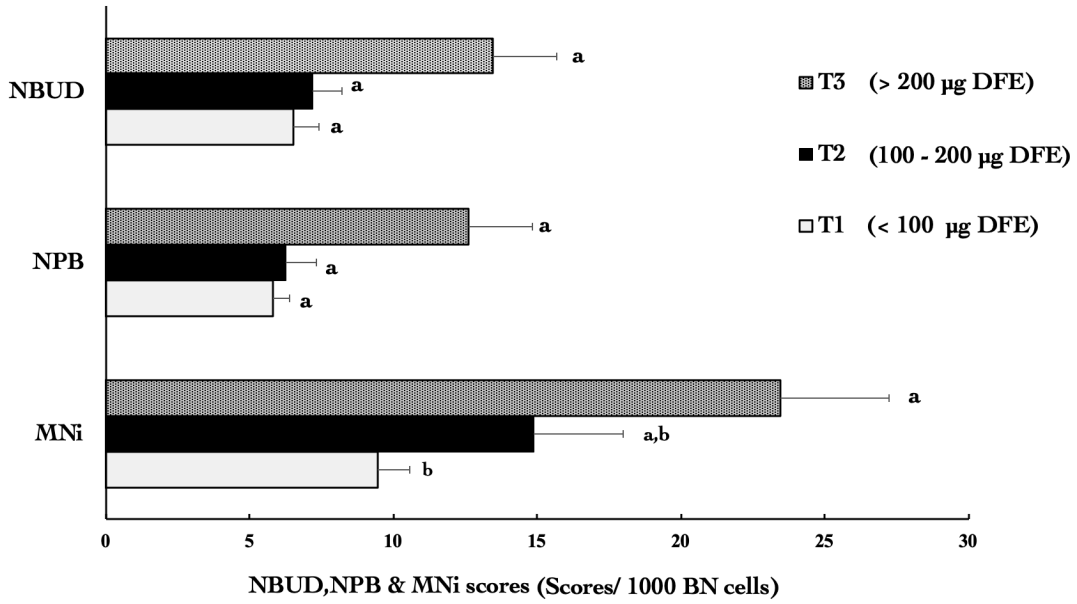


Figure S3.4: Cytome Biomarker frequency in human lymphocytes based on folic acid intake tertiles (Model 3). MNi, NPB & NBUD scores measured by CBMN assay, scores represented per 1000 binucleated (BN) cells. LCL. MNi: micronuclei, NPB: nucleoplasmic bridge, NBUD: nuclear bud. Lymphocytes were isolated from human participants divided into tertiles (T1, T2, T3) based on total folic acid intake. Folic acid intake presented as µg DFE (dietary folate equivalent). Data were presented as mean (± SEM). Values with different superscripts indicate significant differences at $p<0.05$. ANOVA $P<0.001$.

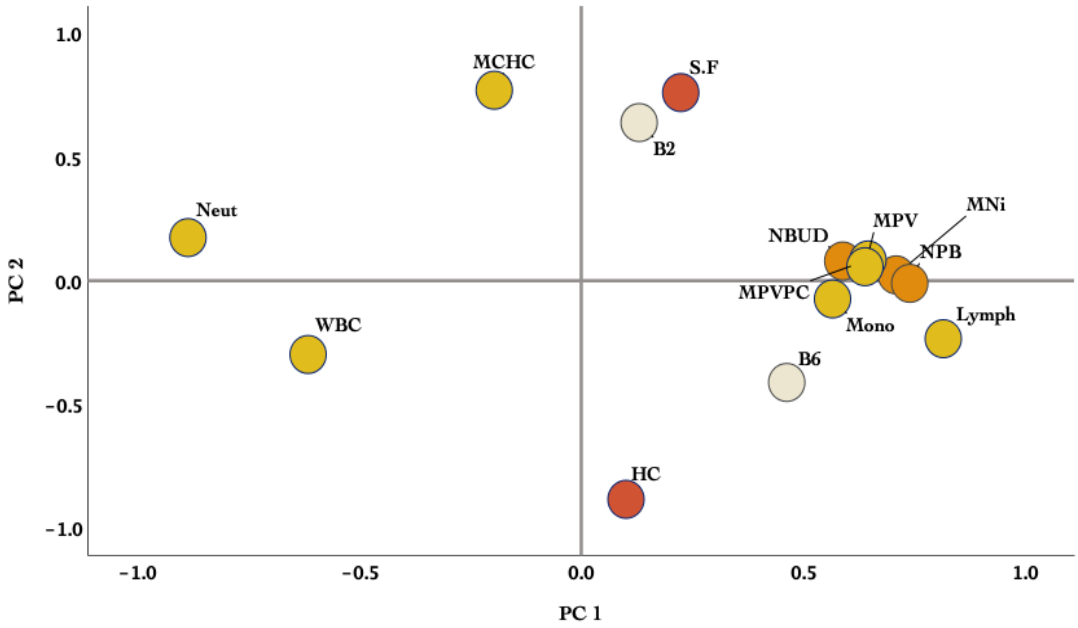


Figure S3.5: PCA loading plot of possible surrogate markers and their relationship with cytome biomarkers. PCA cumulative variance explained (53.1%); PC1 (33.8%), PC2(19.3%), KMO=0.65, p-value < 0.0001. S.F: Serum Folate (µg/L), HC: plasma Homocysteine (µmol/L), B2: plasma Riboflavin (µg/L), B6: plasma Pyridoxal phosphate (µg/L), MCHC: Mean Corpuscular Hemoglobin Concentration (d/dL), MPV: Mean platelet volume (fL), MPV/PC: MPV/ Platelet count ratio. WBC: White blood cells (10^9/L), Neut: Neutrophils (%), Lymph: Lymphocytes (%), Mono: Monocytes (%). MNi: Micronuclei, NPB: nucleoplasmic bridges, NBUD: Nuclear bud.

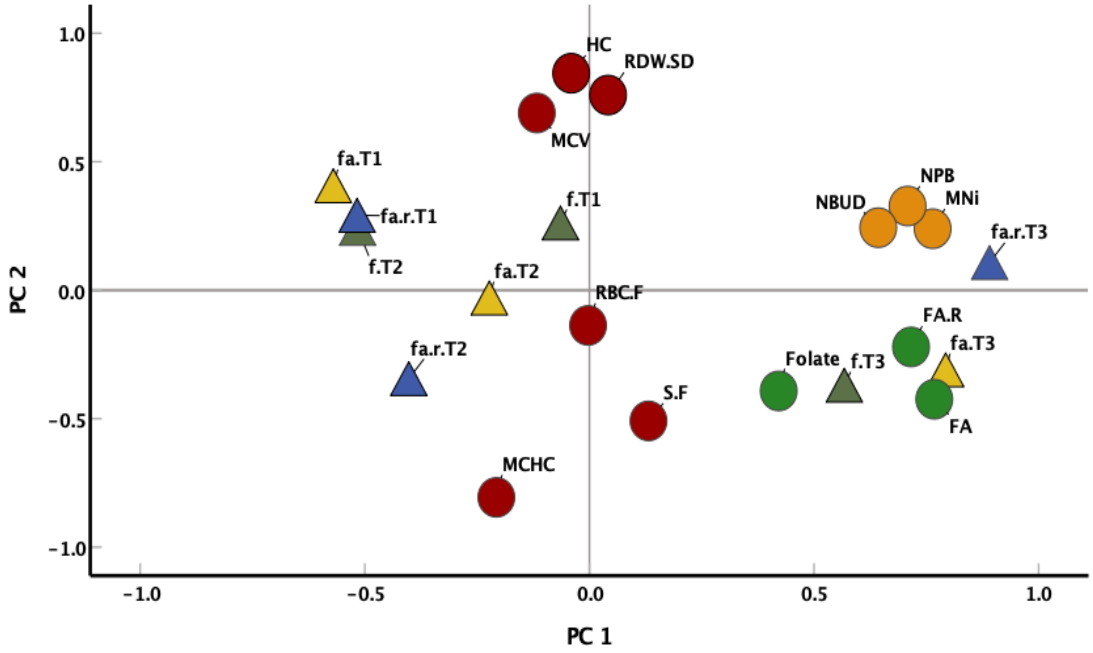


Figure S3.6: PCA loading plot of model tertiles and their relationship with cytome biomarkers and conventional blood markers. PCA cumulative variance explained (63.9%); PC1 (33.9%), PC2 (30.0%), KMO- 0.76, $p < 0.0001$. T1-T3 : tertiles. S.F: Serum folate (ng/µL), RBC.F : Red blood cells folate (ng/ µL). HC: plasma Homocysteine (µmol/L), B2: plasma Riboflavin (nmol/L), MCV: Mean Corpuscular Volume (fL), MCHC: Mean Corpuscular Hemoglobin Concentration (g/dL), RDW.SD: Red cell Distribution Width- Standard deviation (fL). FA: Folic acid from fortified food (µg DEF), FA.R : Folic acid: total food Folate Ratio. MNi : Micronuclei, NPB : nucleoplasmic bridges, NBUD : Nuclear bud, (score/ 1000 BN cells).

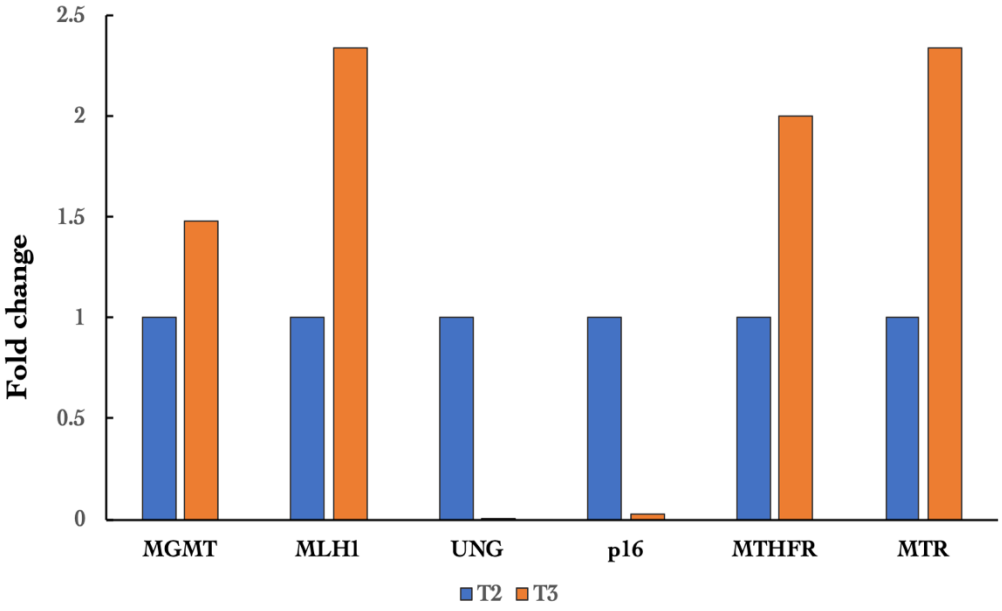


Figure S3.7: Comparison of gene expression profiling between second and third tertile of model 4, based on FAR. Total mRNA was isolated from blood samples of participants divided based on folic acid intake ratio (FAR). T2 < 0.35, T3 > 0.5, n=11. ANOVA= ns.

Table S3.1A: Description of Model 1, serum folate tertiles.

Tertiles (n)	T1(11)	T2(11)	T3(11)	p- value
Serum Folate	9.3 (1.9)	14.4 (1.3)	20.6 (2.8)	< 0.0001
RBC Folate	563.5 (144.7)	571.6 (119.5)	689.9 (210.9)	0.14
Homocysteine	27.4 (7.2)	20.0 (6.2)	16.8 (6.3)	0.002
Folate	576.0 (339.4)	626.3 (336.4)	484.8 (209.4)	ns
Folic acid	204.2 (204.8)	233.7 (169.1)	187.1 (89.6)	<0.0001
Folic acid ratio	0.31 (0.01)	0.36 (0.01)	0.42 (0.02)	ns
MCV	101.3 (7.8)	92.7 (6.4)	89.6 (8.3)	< 0.0001
RDW-SD	51.5 (5.5)	45.3 (3.7)	43.0 (3.6)	0.003
MCHC	30.0 (1.9)	31.4 (1.6)	31.8 (1.3)	0.03

Table S3.1B: Description of Model 2, total folate intake tertiles.

Tertiles (n)	T1(11)	T2(11)	T3(11)	p- value
Folate	289.8 (73.4)	490.5 (59.4)	906.9 (237.9)	< 0.0001
Folic acid	98.6 (53.6)	152.2 (39.1)	374.2 (168.3)	< 0.0001
Folic acid ratio	0.36 (0.03)	0.32 (0.01)	0.41 (0.01)	ns
Serum Folate	13.9 (4.4)	15.4 (4.8)	15.0 (6.3)	ns
RBC Folate	637.2 (215.7)	571.3 (145.1)	616.5 (143.6)	ns
Homocysteine	25.5 (7.7)	19.0 (4.8)	19.6 (9.2)	0.09
MCV	96.8 (7.9)	93.9 (9.3)	92.9 (9.6)	ns
RDW-SD	48.2 (5.6)	45.1 (4.6)	46.5 (6.5)	ns
MCHC	30.2 (1.6)	31.9 (1.6)	31.2 (1.7)	0.07

Table S3.1C: Description of Model 3, folic acid intake tertiles.

Tertiles (n)	T1(11)	T2(11)	T3(11)	p- value
Folic acid	85.6 (35.6)	155.9 (18.5)	383.4 (157.8)	<0.0001
Folate	362.4 (124.2)	503.9 (240.9)	820.7 (298.1)	<0.0001
Folic acid ratio	0.24 (0.01)	0.38 (0.02)	0.46 (0.01)	0.003
Serum Folate	13.9 (4.5)	14.5 (5.2)	15.9 (5.1)	ns
RBC Folate	583.7 (210)	605.6 (146.2)	635.7 (153.9)	ns
Homocysteine	24.1 (7.1)	21.4 (8.3)	18.8 (7.8)	ns
MCV	95.2 (7.2)	97.1 (9.5)	91.3 (9.4)	ns
RDW-SD	47.1 (5.8)	47.2 (4.8)	45.6 (6.4)	ns
MCHC	30.8 (1.9)	31.3 (1.6)	31.1 (1.8)	ns

Table S3.1D: Description of Model 4, folic acid intake ratio tertiles.

Tertiles (n)	T1(11)	T2(11)	T3(11)	p- value
Folic acid ratio	0.2 (0.06)	0.35 (0.04)	0.54 (0.02)	< 0.0001
Folic acid (DFE)	99.1 (51)	180.7 (92.5)	345.1 (184.5)	< 0.0001
Folate (DFE)	492.0 (253.9)	510.2 (240.4)	684.9 (369.1)	ns
Serum Folate	13.6 (4.8)	14.5 (5.2)	16.2 (5.5)	ns
RBC Folate	581.7 (205.8)	625.9 (144.2)	617.3 (168.4)	ns
Homocysteine	23.2 (7.0)	20.5 (7.8)	20.4 (8.9)	ns
MCV	94.7 (6.9)	96.3 (9.5)	92.6 (10.3)	ns
RDW-SD	46.9 (5.5)	46.2 (5.4)	46.6 (6.3)	ns
MCHC	31.4 (1.6)	31.1 (2.1)	30.8 (1.6)	ns

Table S3.2: Mean nutrient intake of study participants.

Nutrient Intake	*N*	*Mean (SD)*	*RDA or AI*[c]	
Protein (g)	31	89.3 (37.2)	0.8 g/kg/day	
Choline (mg)	31	306.5 (130.5)	M: 550 mg/ day	F: 425 mg/day
Iron (mg)	31	14.9 (6.8)	M: 8 mg/ day	F: 18 mg/day
Vitamin B1 (mg)	31	1.31 (0.54)	M: 1.2 mg/ day	F: 1.1 mg/day
Vitamin B2 (mg)	31	1.22 (0.81)	M: 1.3 mg/ day	F: 1.1 mg/day
Vitamin B3 (mg)[a]	31	21.2 (10.2)	M: 16 mg/ day	F: 14 mg/day
Vitamin B6 (mg)	31	1.54 (0.92)	M: 1.3 mg/ day	F: 1.3 mg/day
Vitamin B12 (mcg)	31	4.94 (12.2)	M: 2.4 mcg/ day	F: 2.4 mcg/day
Folate (mcg)	33	401.76 (230.9)		
Folate (mcg DFE) [b]	33	495.9 (303.8)	M: 400 mcg/ day	F: 400 mcg/day

[a] Recommendation is expressed as niacin equivalent (NE)
[b] Recommendation is expressed as dietary folate equivalent (DEF)
[C] Recommendation are expressed as Recommended Dietary allowance (RDA) or Adequate Intake (AI) for group ages (19-50).

Table S3.3: Mean systemic markers of study participants.

Systemic Markers	N	Mean (SD)	Normal Rang	
Serum folate (µg/L) [a]	33	14.8 (5.12)	2 - 20	
RBC folate (µg/L) [a]	33	608.32 (168.4)	140 - 628	
Homocysteine (µmol/L)	33	21.41 (7.82)	4 -15	
Serum B12 (pg/ml)	33	459.5 (186.5)	200 - 950	
MMA (nmol/L)	33	125.7 (49.1)	87 - 318	
Plasma B6 (µg/L)	33	18.9 (15.4)	5 - 50	
Plasma B2 (µg/L)	33	20.21 (23.6)	4 - 24	
HGB (g/dL)	33	14.92 (1.81)	**M**: 13.5 -17.5	**F:** 12 - 15.5
HCT (%)	33	48.1 (5.7)	**M**: 42 - 54	**F:** 38 - 46
RBC (10^6/L)	33	5.10 (0.6)	**M**: 4.7 - 6.1	**F:** 4.2 - 5.4
WBC (10^9/L)	33	6.26 (1.68)	4.5 - 11	
Neut (%)	33	51.6 (10.7)	45 - 75	
Lymph (%)	33	36.4 (8.9)	20 - 40	
Mono (%)	33	8.1 (2.3)	2 – 8	
MCV (fL)	33	94.6 (8.7)	80 - 96	
MCH (pg)	33	29.3 (2.1)	23 - 31	
MCHC (g/dL)	33	31.1 (1.74)	32 - 36	
RDW-SD (fL)	33	46.6 (5.6)	39 - 46	
PLT (10^3/µl)	33	264.3 (63.93)	150 - 400	
MPV (fL)	33	11.19 (0.83)	7 – 11	

[a] to convert to nmol/l multiply by 2.6, µg/L= ng/µL.

Table S3.5: Comparison between RBC folate and RBC indices between genders.

Mean (SD)	RBC Folate	RBC	HGB	HCT	MCV	MCH	MCHC	RDW SD	RDW CV
M (21)	551.3 (122)	5.3 (0.6)	15.6 (1.7)	49.9 (5.1)	93.7 (7.2)	29.3 (1.8)	31.4 (1.7)	45.9 (4.1)	13.4 (1.6)
F (12)	708 (195.6)	4.7 (0.5)	13.7 (1.4)	44.9 (5.3)	96 (11.5)	29.2 (2.6)	30.6 (1.8)	47.9 (7.6)	13.6 (1.12)
P	0.008	0.002	0.002	0.01	0.4	0.8	0.2	0.3	0.8

Table S3.6: Correlation levels of conventional systemic markers related to folate status.

Pearson *r*	Serum Folate	P [a]	Homocysteine	P [a]
RBC Folate	0.36	0.02	-0.26	0.07
Homocysteine	-0.57	<0.001	NA	NA
MCV	-0.43	0.007	0.66	<0.001
MCHC	0.44	0.006	-0.73	<0.001
RDW-SD	-0.55	0.001	0.74	<0.001

a, 1-tail t-test, NA (not applicable).

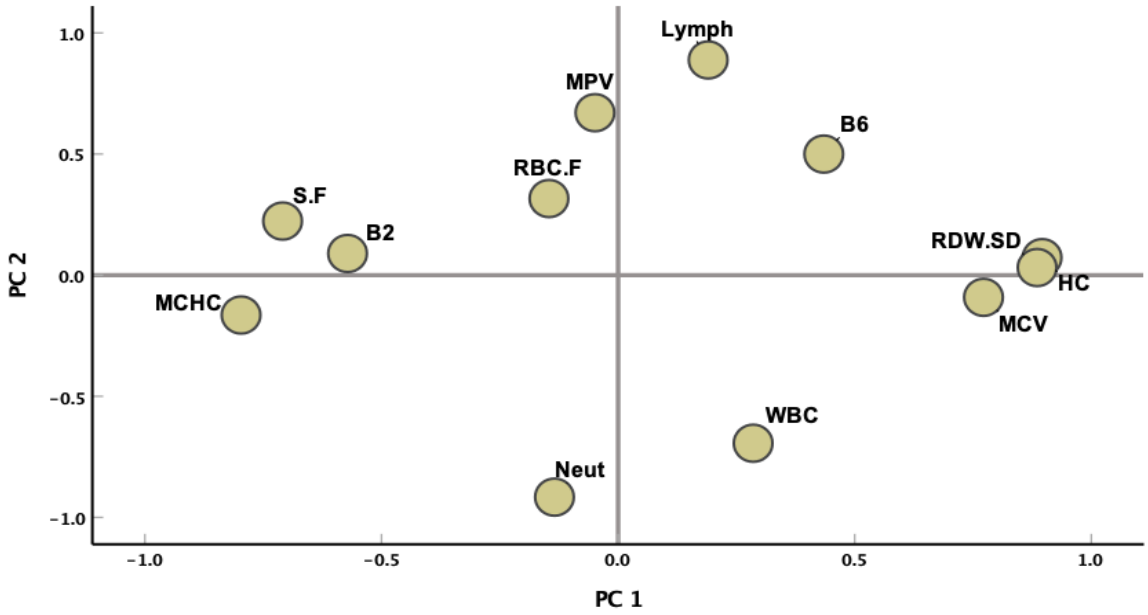


Figure S3.8: PCA loading plot of systemic markers. Cumulative variance explained (58.4%); PC1 (33.6%), PC2(24.8%), KMO=0.7, p-value < 0.0001. RBC.F: Red Blood Cells folate (µg/L), S.F: Serum Folate (µg/L), HC: plasma Homocysteine (µmol/L), B2: plasma Riboflavin (µg/L), B6: plasma Pyridoxal phosphate (µg/L), MCV: Mean Corpuscular Volume (fL), MCHC: Mean Corpuscular Hemoglobin Concentration (g/dL), RDW-SD: Red cell Distribution Width- Standard deviation (fL). MPV: Mean platelet volume (fL), WBC: White blood cells (10^9/L), Neut: Neutrophils (%), Lymph: Lymphocytes (%).

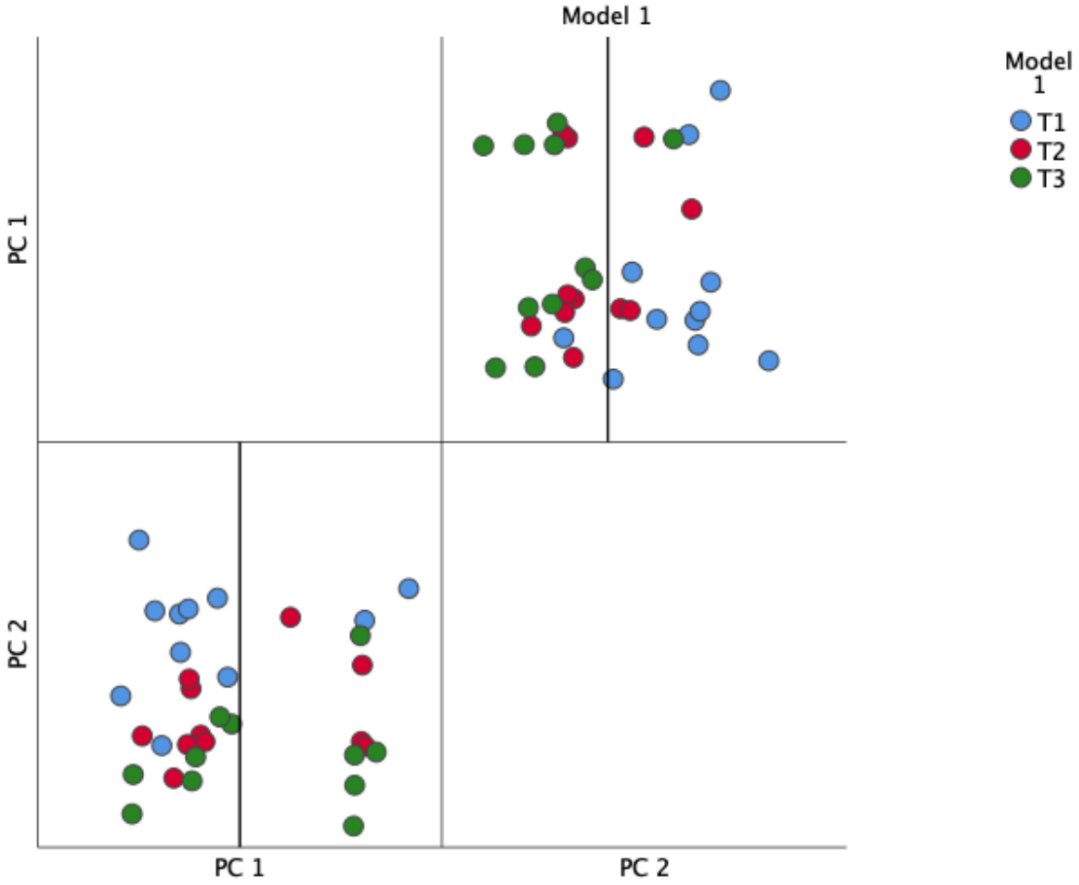


Figure S3.9: PCA scoring plot for Model 1. PC1 (38.0%), PC2 (25.7%), KMO= 0.72, p-value < 0.0001.

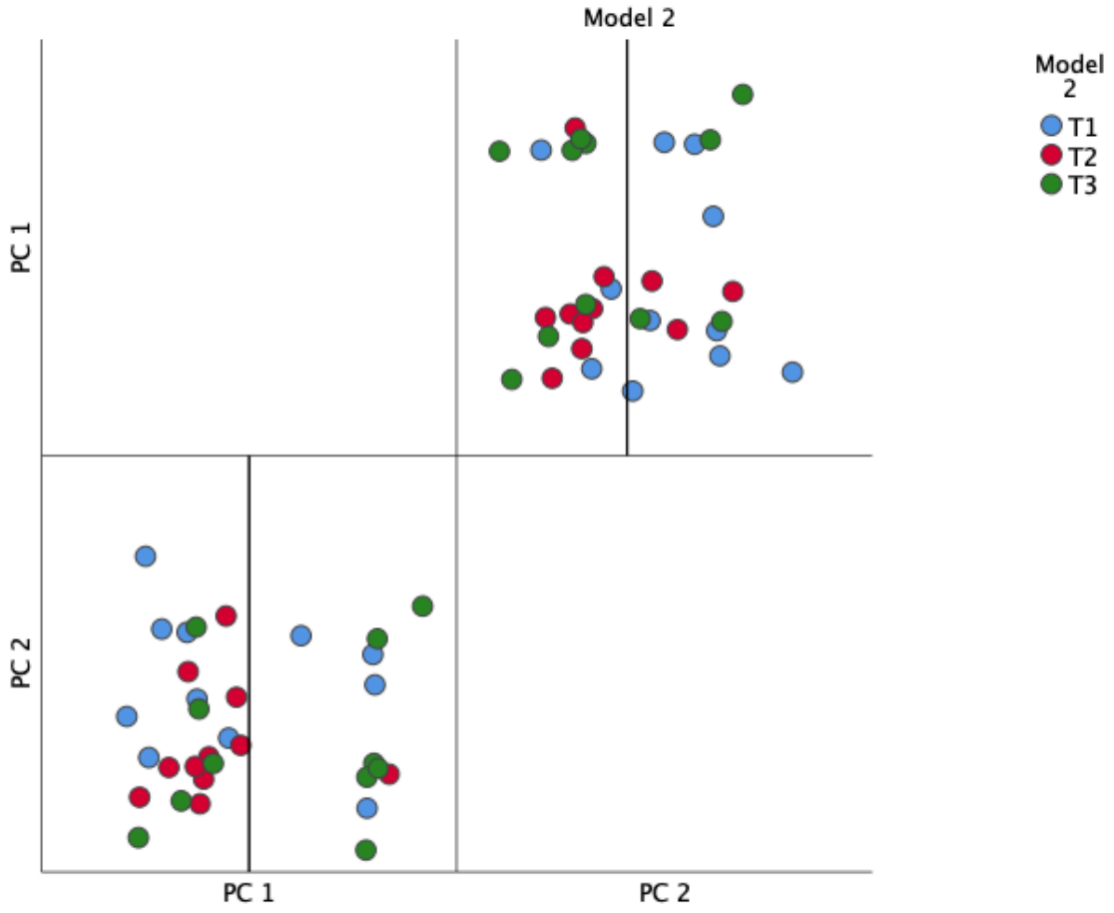


Figure S3.10: PCA scoring plot for Model 2. PC1 (33.0%), PC2 (26.8%), KMO= 0.64, p-value < 0.0001**.**

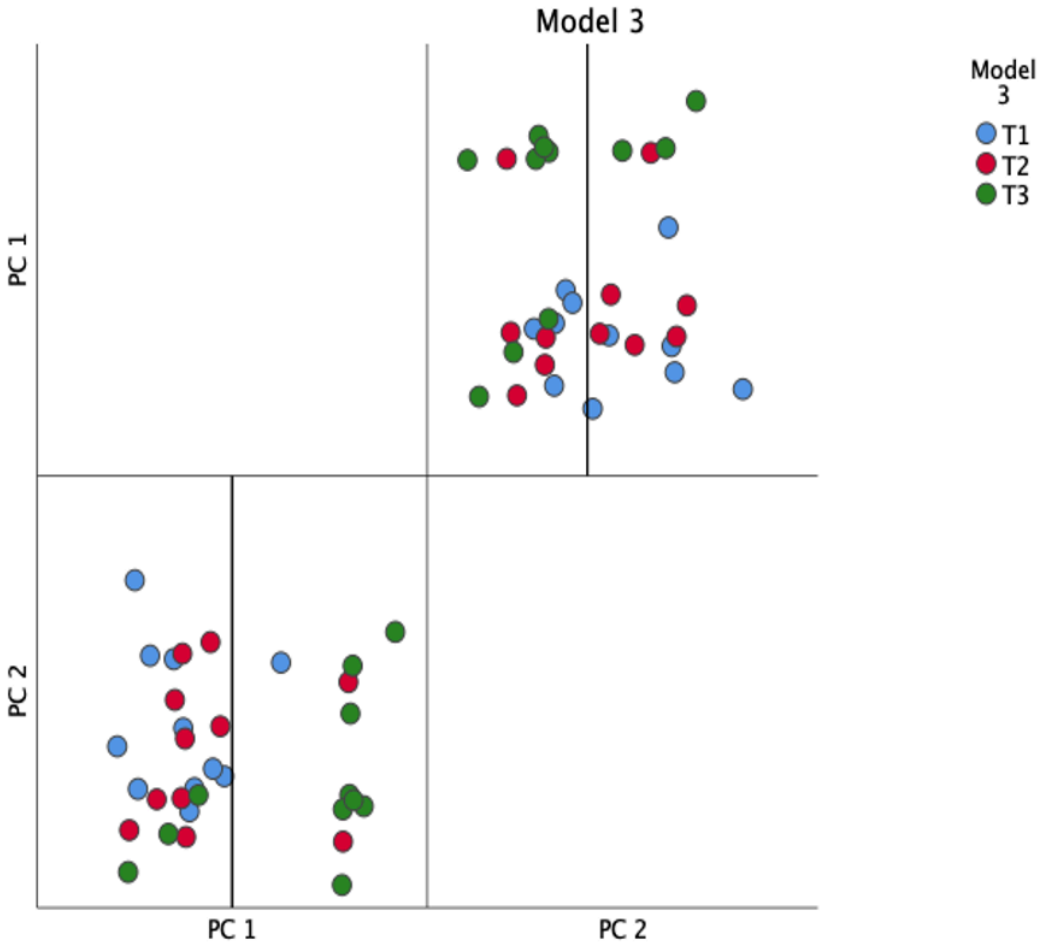


Figure S3.11: PCA scoring plot for Model 3. PC1 (35.0%), PC2 (27.8%), KMO= 0.72, p-value < 0.0001.

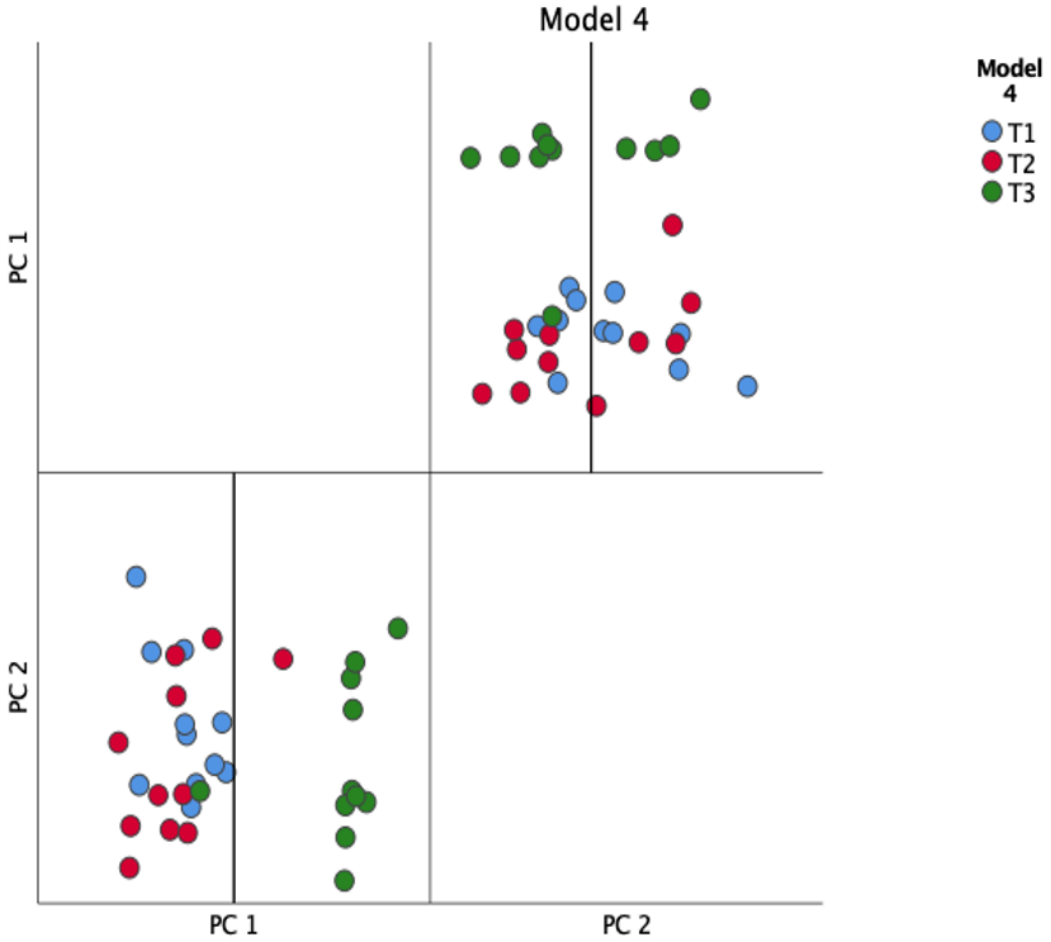


Figure S3.12: PCA scoring plot for Model 4. PC1 (33.9%), PC2 (30.0%), KMO= 0.76, p-value < 0.0001

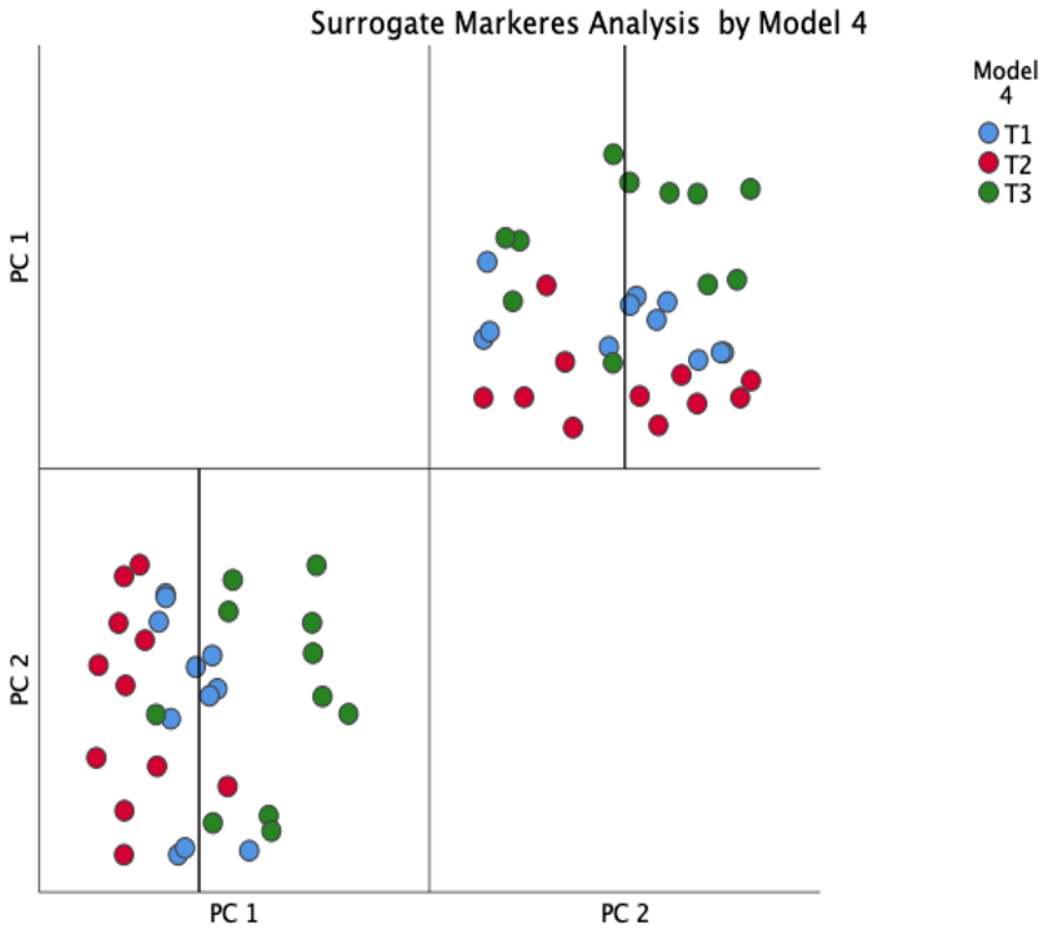


Figure S3.13: PCA scoring plot for surrogate markers analysis by Model 4. PC1 (36.7%), PC2(16.9%), KMO=0.69, p-value < 0.0001.

CHAPTER 4: SUMMARY & FUTURE DIRECTION

The finding of this study supports several concerns raised about the safety of folic acid (FA), especially at high doses. Folate deficiency (FD) is known by the accumulated evidence to induce DNA damage and carcinogenesis by several mechanisms. In addition to several reports concerning FA and its safety at higher doses, the presence of unmetabolized FA (UMFA) in the blood, which linked to several deleterious effects, led to test the hypothesis that:

a) high doses of FA could lead to functional FD and consequence DNA damage. Using the in vitro model, our data showed that FA has genotoxic effects to the cells. We observed higher micronuclei (MNi), nucleoplasmic bridges (NPB) and nuclear buds (NBUD) formation which indicates high genome damage. The pattern of damage observed in cytome biomarkers is similar to FD.

b) excess FA intake through consumption of fortified food is associated with higher cytome biomarkers, i.e., greater genome damage. Our results indicated that the highest tertile of FA intake (> 200mcg) is associated significantly with an increase in cytome biomarkers compared with the lowest tertile (< 100 mcg).

c) the natural form of folate would diminish the consequent damage of excess FA. Our *in vitro* model showed the direct impact of excess FA (synthetic form) in media on genome stability. It is hard to measure the impact of 5-mTHF in cell culture models due to its low stability in the environment (media). Therefore, in the human study we evaluated the association between FA ratio to natural folate as well. We found that the higher the ratio (FAR > 0.5), the higher the damage (higher cytome biomarkers), albeit

the lower the ratio (FAR < 0.35), the lower the cytome biomarkers. This association suggests that the natural folate has a protective property against the genotoxic effects of the synthetic form.

Given that we only observed this association using food diary intake, more controlled intervention studies are needed to confirm our results. For future studies, we need to consider several points: **a)** the high variability of dihydrofolate reductase (DHFR) activity in humans and methylenetetrahydrofolate reductase (MTHFR) genotypes; **b)** the ratio of FA intake to natural form; **c)** the amount of natural folate contributed from gut microflora (but less is known about this subject); and **d)** the need to use alternative tests to evaluate folate status other than red blood cell (RBC) and serum folate and homocysteine. For example, the histidine load test (FIGLU-test) could be useful in the determination of the availability of tetrahydrofolate (THF).

Our results in no way indicate that the fortification program is harmful and should be terminated. We acknowledge the importance of the program for the target high-risk population that the program is intended to help. However, with our current knowledge and advanced tools, the program should be re-evaluated. There are deep concerns about the prolonged and high exposure of other vulnerable populations to FA, such as cancer patients. We join our voice with others who question the safety of the current program. We think several steps can be taken in this regard: **a)** mandate new regulation, such as: targeting lower aim of fortification with FA, setting an upper limit for added FA in manufactures, and limiting types of food that could be fortified; **b)** increasing public awareness about FA and including the information and quantity of FA in food labeling;

c) finding alternative ways to fortify food, for example, fortifying food with Nano - encapsulated L-methyl folate or fortifying dairy products with some engineered probiotics that produce a higher amount of natural folate in the colon through more researches in this regard [*4*]; **d)** finding other parameters to monitor the program, other than RBC folate, serum folate or homocysteine levels; **e)** perform mass-survey research to target the food that contributes mostly to UMFA, and develop more validated food folate surveys other than the DFE Block Survey.

The need to develop this work is not only related to the genotoxic effect of FA or carcinogenesis. Other concerns should be addressed as well. For instance, there is a higher chance that excessive FA intake exceeding the upper limit can disguise B12 deficiency and consequent cognitive impairments. The deleterious effect on mental health and development cannot be reversed. This concerns for child development as well as that of the elderly population. It was shown that 5-mTHF is the only form of folate that can cross the blood-brain barriers [108], and with B12 deficiency, this issue is exacerbated. Therefore, more work should be done to investigate the role of high FA intake on health.

APPENDIX A

(Food-Folate Survey; Screening Tool)

Q1: How do you describe your diet style?

☐ Vegan ☐ Vegetarian ☐ Protein-rich ☐ Typical

Q2: How do you describe your daily food intake?

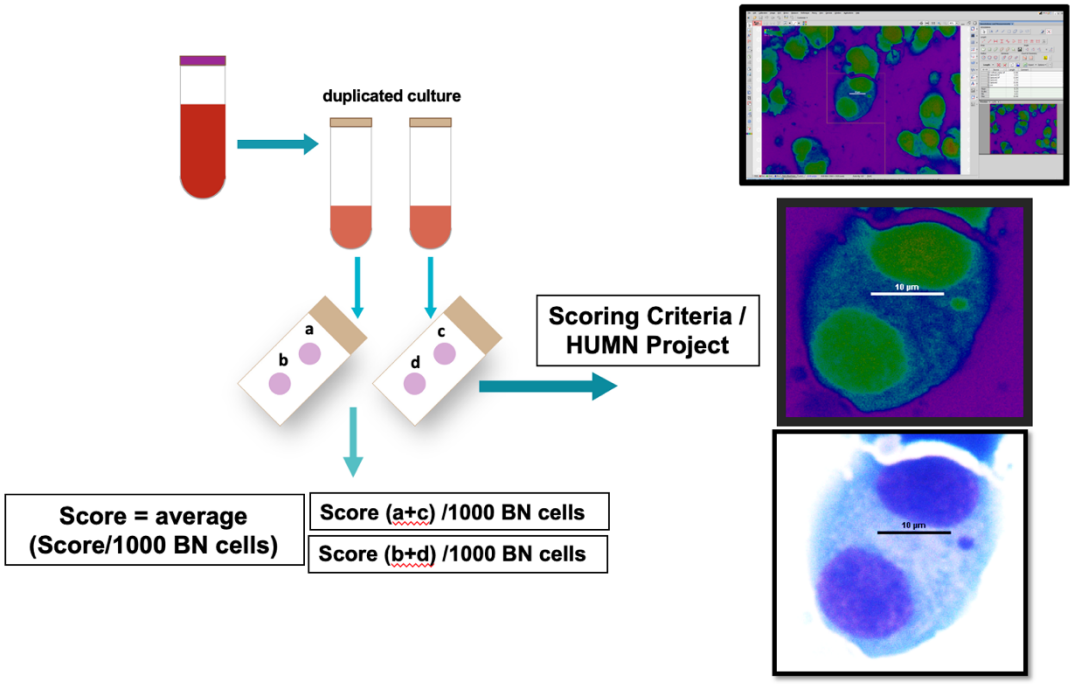
☐ Mostly Carbs. ☐ Mostly proteins ☐ Mostly fruit and vegetables ☐ Balanced

Q3: How often do you consume these foods per week?

Foods	None	1X	2-3X	≥ 4 X	Can you tell the amount?
Ready to eat breakfast					
Pancakes, waffles					
Breakfast cereals					
Oatmeal					
Cakes, muffin, croissant, cookies, crackers					
Granola					
Bread (white – whole wheat)					
Bagels, tortillas, English muffin					
Pasta, Rice					
Meats					
Livers (chicken, turkey, veal, lamb, beef, pork)					
Beans					
Lentil, Humus					
Milk					
Orange juice					
Potato					
Oat, flour, cornmeal					
Asparagus, Artichoke					
Broccoli, beets, turnip, Brussel sprouts					
Spinach, lettuce, avocado					
Nutritional yeast					
Energy bars, protein bars, sank bars, breakfast bars					Brand?
Complete nutrition drinks (special K, Ensure, Boost, Orgain, Carnation, …. others)					Brand?
Multivitamin supplements, Prenatal supplements					Brand?
Folic acid supplements, B-vitamins supplements					Brand?

APPENDIX B

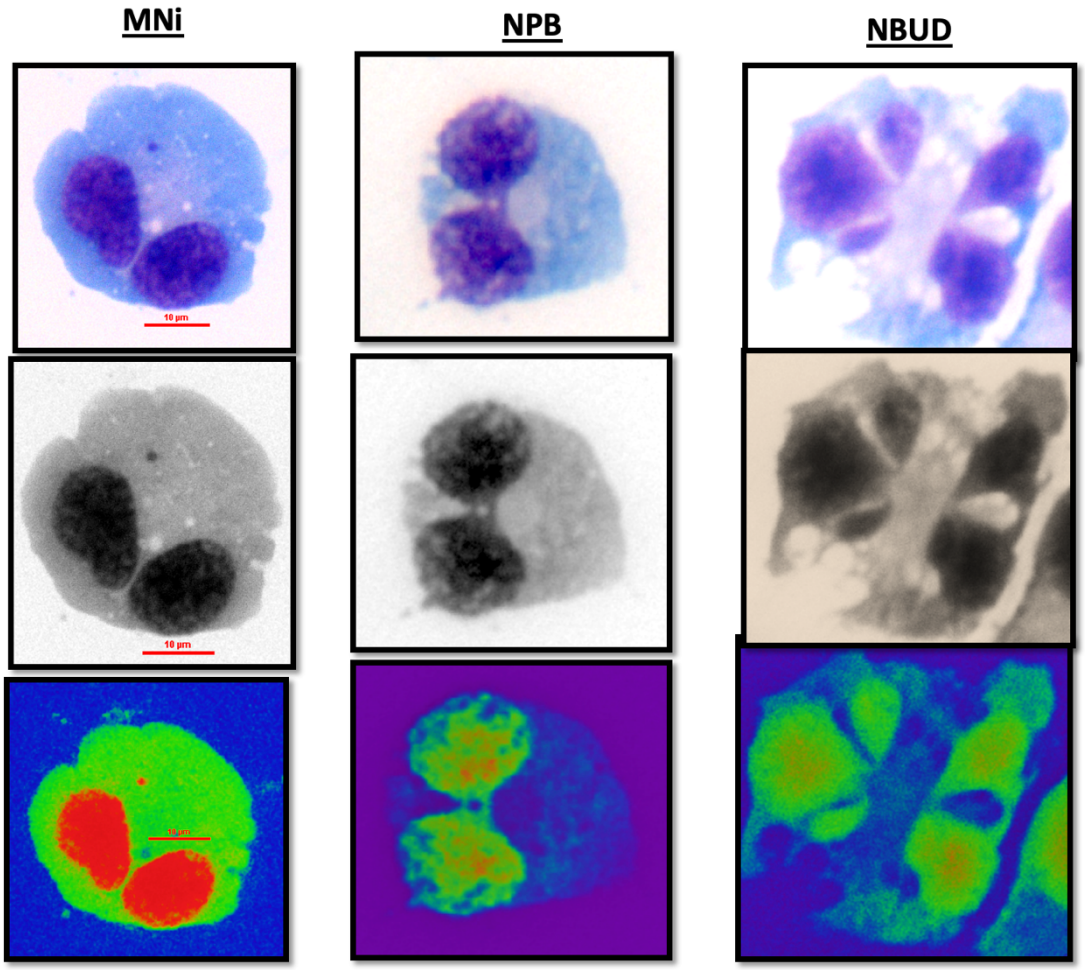
(Sampling and Scoring Plan for CBMN Assay)



A schematic showing the study approach for sampling and scoring for cytokinesis - blocked micronucleus (CBMN) assay. The plan enables an estimation of experimental variation and scorer bias, following the criteria of Fenech (2007) [2].

APPENDIX C

(Cytome Biomarkers)



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ABSTRACT

THE RELATIONSHIP BETWEEN EXCESSIVE FOLIC ACID INTAKE AND GENOME INSTABILITY IN HUMAN LYMPHOCYTES.

by

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August 2020

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Major: Nutrition and Food Science

Degree: Doctor of philosophy

Since its synthesis in 1945, Folic acid (FA) is widely used as a supplement/additive to our food due to its stability and high bioavailability. FA is proposed to alleviate anemia and reduce neural tube defects (NTD). As a provitamin, FA is activated through a two-step reaction catalyzed by dihydrofolate reductase (DHFR) in the folate metabolism pathway. FA activation rate is found to be slow in humans. After the implementation of mandatory food fortification program of grain and grain products with folic acid in the USA in 1998, many concerns have been raised about the unintended deleterious consequence of exposing the whole population to vast amounts of folic acid. These concerns have been further escalated after several studies reported that upon fortification, certain human populations are exceeding the UL intake of FA, resulting in an increase in cancer rate (e.g., colon, stomach, and breast cancer) associated with an increment in mortality rate. Furthermore, recent studies have proposed the U-shape effect of folic acid on the onset and progression of cancer. In this study, we analyzed the correlation between the high intake of folic acid and the level of genome damage in lymphocytes by utilizing

the CBMN cytome assay in human and cell culture models, and their relation to folate systemic markers. We hypothesized that the chronic and excessive exposure of folic acid via fortified food consumption induces functional folate deficiency.

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