The effect of excess folate intake on immune function and peripheral neuropathy associated with transcobalamin II polymorphism

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Dedication

To my parents (all four of them), Prayoon Sawaengsri, Vanna Heflin, Boonroeng Bowers, and Lewis Bowers, who have always supported me and were understanding when I kept explaining that I wasn't sure when I'd be done and finally get a job. They always wanted me to be a doctor, even if it's not a medical doctor.

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Abstract

Adequate folate intake is necessary for health throughout life. However, previous research showed that excess folic acid, the synthetic form of folate used in supplements and fortified foods, may have harmful effects including worsening clinical symptoms of vitamin B-12 deficiency and decreasing natural killer cell (NK) cytotoxicity in women older than 60 years. NK cells are innate immune cells important for defense against cancerous and virally infected cells and impairment in their activity increases disease risk. The central hypothesis of this project is that excess folic acid impairs NK cytotoxicity, increases severity of influenza infection in aged female mice, and exacerbates clinical symptoms of vitamin B-12 deficiency in the presence of the 776C>G polymorphism in the vitamin B-12 transporter transcobalamin II (TCN2).

In the first aim, we used a mouse model of excess folic acid intake to determine the effects on natural killer cell cytotoxicity. We fed older (14-16 mo) female C57bL/6 mice a control (1xRDA) or high folic acid diets (20xRDA) for 3 months. We found that aged female mice fed a high folic acid diet had reduced NK cell cytotoxicity in spleen ($P<0.04$), lower mature cytotoxic /naïve NK cell ratio ($P=0.03$), decreased production of lipopolysaccharide stimulated interleukin (IL)-10 secretion ($P<0.05$) compared to mice on control diet. The difference in NK cell cytotoxicity between dietary groups was abolished when the splenocytes were supplemented with exogenous IL-10 prior to assessment of the NK cytotoxicity, suggesting that the reduced NK cell cytotoxicity of the high folic acid group was at least partially due to reduced IL-10 production.

The second aim of the proposed project was to determine the effect of excess dietary folic acid on the severity of disease in an influenza infection model of mice. Female 16 month old C57bL/6 mice were fed a control (1xRDA) or high folic acid diet (20xRDA) for 3 months before they were infected with H1N1 influenza virus for 2 or 5 days of infection. We found that aged female mice fed a high folic acid had increased viral titer 2 days after infection and reduced inflammatory cytokine gene expression of IL-6, IL-1β, and interferon-gamma (IFN-γ) 5 days after infection in lung cells. These cytokines are important for viral clearance in the lung. There were no differences in weight loss, lipopolysaccharide stimulated cytokine secretion or cytokine protein in lung supernatant.

In addition to effects on immune system, high intakes of folic acid may worsen clinical symptoms of vitamin B-12 deficiency. The TCN2 polymorphism of the vitamin B-12 transport protein transcobalamin II (TCN2) gene is associated with reduced bioavailability of vitamin B-12 to tissue. In the third aim, we determined if excess folate intake increased odds of peripheral neuropathy in the presence of the TCN2 776C>G polymorphism in a cross-sectional study of homebound elders in the Nutrition, Aging, and Memory in Elders Study (NAME). We found that odds for peripheral neuropathy were 3 fold higher for GG genotypes when compared to CC genotypes (OR: 3.33; 95% CI: 1.15, 9.64). When folate intake was above twice the recommended dietary allowance (800 μg/day), GG genotypes had 6.9 fold higher odds for peripheral neuropathy compared to CC genotype (OR: 6.9; 95% CI: 1.31, 36.36) but not when folate intake was ≤800 μg (OR: 1.5; 95% CI: 0.18, 12.33).

The first two aims demonstrated that excess folate intake led to reduced NK cell cytotoxicity and increased influenza infection. The third aim showed that TCN2 776C>G polymorphism was associated with increased odds for peripheral neuropathy in elderly despite normal vitamin B12 status, especially if their folate intake was in excess of twice the recommended dietary allowance. Overall, these results demonstrate that excess folate may be harmful to the aging population and that further studies are needed to elucidate the mechanisms.

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Introduction

Statement of the Problem and Significance

Statement of Hypothesis

Statement of the Problem and Significance

The United States mandated folic acid fortification of flour and grain products in 1998 to decrease incidence of neural tube defects. Folic acid, the synthetic form of folate used in supplements and fortified foods, must be converted to tetrahydrofolate by dihydrofolate reductase (DHFR) before it can be used in the folate pathway. The capacity of DHFR to metabolize folic acid can be exceeded with excess folic acid from multivitamin use and the consumption of fortified food [2], which results in the appearance of unmetabolized folic acid in plasma [3, 4]. Approximately 39% of the U.S. population consumes multivitamin pills containing folic acid, with the majority being women over the age of 60 [5, 6].

Studies from our group showed that presence of unmetabolized folic acid in plasma, indicative of high folic acid intake, is associated with decreased natural killer cell (NK) cytotoxicity in women older than 60. NK cells are innate immune cells important for defense against cancerous and virally infected cells and impairment in their activity increases disease risk. Thus, the finding that unmetabolized folic acid in plasma is associated with decreased NK cytotoxicity in older women may have negative implications for cancer development and susceptibility to infection in older populations.

In addition to the effects of excess folic acid on immune function, high intakes of folic acid in conjunction to low intakes of vitamin B-12 have led to increased risk for clinical symptoms associated with vitamin B-12 deficiency such as peripheral neuropathy and anemia. The 776C>G polymorphism of the vitamin B-12 transport protein transcobalamin II gene (TCN2) is associated with reduced bioavailability of vitamin B-12 to tissue. It is not known whether excess intake of folic acid can worsen clinical outcomes in the presence of the polymorphism.

The overall objective of this project is to determine the effects of excess folate intake on innate immune function and if clinical outcomes associated with vitamin B-12 deficiency is worsened in the presence of the *TCN2* 776C>G polymorphism.

Statement of Hypothesis

The central hypothesis of this project is that excess dietary folic acid will have adverse effects on health, including the innate immune system, and with low vitamin B-12 status. Specifically, we hypothesize that excess dietary folate consumption will decrease NK cell cytotoxicity in aged mice and increase susceptibility and severity of influenza infection. Excess folate intake will also worsen clinical outcomes associated with vitamin B-12 deficiency including those with the *TCN2* 776C>G polymorphism. These hypotheses will be tested through the following specific aims:

Specific Aim 1: To determine the causal relationship between excess folic acid intake and decreased NK cell cytotoxicity using an aged mouse model.

Specific Aim 2: To determine if excess folic acid intake will increase susceptibility and severity of H1N1 influenza infection in an aged mouse model.

Specific Aim 3: To determine the association of the TCN2 C776G polymorphism with clinical symptoms of vitamin B-12 deficiency such as peripheral neuropathy using cross sectional data from the Nutrition, Aging, and Memory in Elders (NAME), a Boston cohort of homebound elderly individuals > 60 years old. We will also determine if these relationships are modified by a high folate intake.

Chapter I

Literature Review

Excess Folate Intake on Immune Function and Vitamin B-12 Availability

Folate

Folate is a water soluble B vitamin essential for health throughout life. The term folate is an umbrella term for a class of molecules consisting of pteridine, p-aminobenzoic acid, and glutamic acid [7]. It plays an important role in one-carbon metabolism which includes synthesis of thymidylate, purines, and the many methylation reactions including those of protein and DNA. Specifically, folate compounds accept, transfer, and modify one-carbon molecules with different forms of folate used for different carbon transactions [7]. However, the main folate form in human plasma and tissues is methyl tetrahydrofolate (mTHF). Adequate folate intake is recognized to be important for the prevention of neural tube defects (NTDs) and other congenital defects [8-11], anemia, and the reduction of risk for many chronic illnesses including cancer [12]. Folic acid is the synthetic form of folate that is fully oxidized and is used commercially in supplements and fortified food due to its stability and bioavailability compared to natural forms of folate.

Folate Fortification

Strong evidence for a protective effect of periconceptional folic acid supplementation against NTDs [8-11] led to the US Public Health Service in 1992 to recommend all women of reproductive age to consume 400μg folic acid daily from supplements or fortified foods [13]. However, only 29% of US women complied with these recommendations [14]. In 1996, the US Food and Drug Administration mandated that all flour and uncooked cereal-grain products be fortified with folic acid folic (140 μg /100 g) by January 1998 [15]. The amount of fortification in the US was expected to increase the average folic acid intake by 100 μg /d, although studies have indicated the increase to be closer to 200μg /d [16, 17]. In addition to consumption of fortified foods, approximately 39% of the U.S. population consumes multivitamin pills

containing folic acid, with the majority being women over the age of 60 [5, 6]. In the 5th and 6th examination of the Framingham Offspring Cohort Study, the prevalence of individuals with folic acid intake above the upper tolerable intake of 1000 μg folic acid/d in supplement users increased from 1.3% to 11.3% [16]. This increase in folic acid consumption has led to concerns about the safety of persistent exposure to folic acid especially in light of studies suggesting possible negative effects of excess folic acid.

Folate Absorption and Metabolism

Both folic acid and reduced folates are absorbed in the jejunum by a saturable, carrier-mediated, pH and energy dependent transport mechanism by equal measures [18, 19]. At physiological doses below the RDA of 400 μg/day, dihydrofolate reductase (DHFR) in the intestinal mucosa converts folic acid to dihydrofolate, which is then reduced by DHFR to tetrahydrofolate before it is methylated to 5-methyltetrahydrofolate (5-MTHF), the form of folate predominantly found in circulation [19]. Unmetabolized folic acid goes to the liver, where some circulating folic acid is removed or converted to 5-MTHF by liver DHFR [19-21]. However, Bailey and Ayling used human liver extract to show that folic acid is not a good substrate for DHFR in humans, with a conversion rate to THF 1300 times slower than with DHF as a substrate [2]. The investigators also showed that rat liver DHFR was on average 56 times faster than human liver DHFR when folic acid is used as the substrate. Using these rates, the researchers identified that human liver containing more than 331 μg of folic acid would lead to saturation of DHFR and result in unmetabolized folic acid in plasma. In addition, Bailey and Ayling showed that the rate of DHFR in humans had a 5-fold variation, suggesting that there is a great variability in the ability of people to metabolize folic acid.

Patanwala et al. conducted a small crossover study in humans to determine the conversion of stable-isotope-labeled folic acid or 5-FormylTHF to 5-MTHF across the intestinal mucosa by sampling blood from the portal and peripheral veins of patients with transjugular intrahepatic porto systemic shunt (TIPSS) in situ [22]. The investigators found that approximately 80% of folic acid is unmodified in the portal vein 15 minutes after ingesting 220 μg folic acid equivalent while only 4% of 5-FormylTHF was found in the portal vein after ingestion of a similar dose. The authors concluded that the human intestine could efficiently convert reduced forms of folate to 5-MTHF, but had limited ability to reduce folic acid.

A study by Kelly and coauthors looked at acute serum response of folic acid with the consumption of folic acid at different doses and in different media [4]. They found that there was a threshold intake of 266 μg of folic acid per meal before unmetabolized folic acid was seen in the plasma when measured 2.25 hours after the meal. Considering the recommended dietary allowance of folic acid is 400 μg/day, this recommendation would lead to unmetabolized folic acid in plasma in some people. Consumption of fortified cereals and multivitamin supplements in addition to refined grains can lead to intakes higher than the tolerable upper intake level of 1000 μg/d. Kalmbach et al. measured circulating folic acid in the Framingham Offspring Cohort before and after fortification and found that folic acid fortification has led to significantly increased unmetabolized folic acid in blood of participants [3]. A recent study by Pfeiffer et al. showed that >95% of participants measured in the NHANES 2007-2008 cohort had detectable unmetabolized folic acid [23].

Taken together, these studies suggest that intakes at even the RDA concentration of 400 μg/day may lead to unmetabolized folic acid in plasma due to saturation of the enzyme DHFR to

convert it to DHF and eventually to THF. The next question is whether excess folic acid intake is harmful.

Excess Folic Acid – Good or Bad?

Despite the health benefits of adequate folate status, there continues to be a concern that excess folic acid may have adverse effects in subpopulation groups not originally targeted for fortification. The Food and Nutrition Board of the Institute of Medicine established a tolerable upper intake level of 1mg/d of folic acid from supplements or fortified foods for adults primarily to avoid masking the neurological symptoms of vitamin B12 deficiency [24]. Excess dietary folic acid has been shown to be associated with increased risk for cancer development, especially in the case of enhancing the development and growth of pre-existing, undiagnosed tumors [12, 25-28]. However, folic acid may influence cancer development even before malignant tumors form. A recent study showed that presence of unmetabolized folic acid in plasma is associated with decreased natural killer cell activity, which is important for screening and killing cancerous cells [1]. In addition to the effects on cancer and the immune function, excess intake of folic acid is also associated with worsened clinical symptoms of vitamin B12 deficiency in the elderly such as cognitive impairment, anemia, and neurological diseases [29].

Excess Folic Acid and Increased Risk for Cancer

In support of the hypothesis that excess folic acid may increase risk for certain cancers, a prospective study by Larsson et al. showed that Swedish women who took multivitamins had increased risk of breast cancer [26]. Stolzenberg-Solomon et al. found that women who reported taking supplemental folic acid greater than 400 μg/d had higher risk of post-menopausal breast cancer than those who reported no supplement use [28]. Mason et al. report a temporal association of colorectal cancer with folic acid fortification [27], and a randomized control trial

showed higher risk of having 3 or more colorectal adenomas with 1 mg/d of folic acid [25]. Excess folic acid may increase risk for cancer via two mechanisms: increasing growth of pre-existing malignant tumors; or inhibition of innate immune function so there is less defense against the initial development of cancerous cells. Because NK activity is important for both viral infections as well as cancer, we hypothesize that reduced NK cell activity associated with unmetabolized folic acid (indicative of high folic acid intake) would also increase susceptibility to infection in older individuals.

Excess folic acid and immune function

We previously showed that presence of unmetabolized folic acid in women older than 60 was associated with decreased natural killer cell (NK) cytotoxicity [1]. The study recruited postmenopausal women in the Seattle area and found that women who consumed a diet low in natural forms of folate (<233 μg/d) and used folic acid containing supplements (< 400 μg/d) had increased NK cell cytotoxicity compared to the reference group of women who consumed low natural folate concentrations (<233 μg/d) and took no supplements (P=0.01). However, women who consumed a folate rich diet (>233 μg/d) and supplemented with > 400 μg/d of folic acid had significantly reduced NK cell cytotoxicity compared to the referent group (P=0.02). This implies that folic acid supplementation may be beneficial in those with low intakes of natural dietary folate, but not in those with already high intakes of natural dietary folate. If folic acid is consumed in greater quantities than what can be reduced to 5-MTHF by DHFR, this would lead to the presence of unmetabolized folic acid in the plasma. The same study showed that presence of any unmetabolized folic acid in the plasma was associated with decreased NK cell cytotoxicity (P=0.04). However, the strong inverse association between unmetabolized folic acid and NK cell cytotoxicity was found in women between 60-75 years old (P=0.002) but not in

women 50-59 years old. This finding suggests the effects of excess folic acid and unmetabolized folic acid on NK cell cytotoxicity is modified by age.

In contrast, another study that looked at Italians ages 90-106 y did not find a cross-sectional association between total folate concentration in plasma and NK cell cytotoxicity[30]. However, Italy does not have mandatory folic acid fortification and thus Italians do not have similar levels of intakes to countries that do fortify.

Taken together, these studies show that high folic acid intake is associated with reduced NK cell activity in older postmenopausal women. However, the main study that supports the association is cross sectional and further studies are needed to prove a causal relationship.

Natural Killer Cells

NK cells are bone marrow-derived lymphocytes that have both cytotoxicity and cytokine-producing effector functions [31]. They are innate immune cells capable of lysing virally infected cells and cancerous cells without prior sensitization, termed natural killing [32]. NK activity is regulated by a balance of activating and inhibitive signals that determine whether or not they are activated to kill target cells. NK cells have activating receptors such as natural killer group 2, member D (NKG2D) and Toll-Like Receptors (TLR) that can detect the presence of distress ligands. They also have inhibitory receptors that interact with major histocompatibility complex (MHC) class I molecules that are constitutively expressed by most healthy cells [31]. When cells become stressed, they lose the MHC class I molecules on the cell membrane. The lack of negative inhibition allows NK cells to bind and kill the stressed cell by releasing pore-forming protein perforin to puncture the target cell membrane, and granzyme serine proteases to activate caspase pathways in the target cells which leads to cell death [33]. This equilibrium

between activating and inhibiting signal ensures the tolerance to self while allowing toxicity towards sick or infected cells.

Natural Killer Cell Maturation

Natural killer cells develop in the bone marrow but differentiate and mature further in the periphery. In mice, all NK cells expressed NK1.1, NKG2D, and NKp46 surface markers and can be subdivided into subsets using the surface markers CD11b and CD27 [34-37]. NK cells differentiate in the following sequence: CD11b-CD27- (DN subset) are immature NK cells with no effector functions; CD11b-CD27+ (R1 subset) are immature NK cells with reduced effector functions; CD11b+CD27+ (R2 subsets) are mature NK cells with the most effector functions such as increased cytotoxicity and cytokine secreting activity; and CD11b+CD27- (R3 subsets) are terminally differentiated, mature NK cells that predominantly secrete cytokines [34, 37]. Chiossone et al. determined the kinetics of NK cell development in NK depleted mice and found that DN and R1 subsets peaked at Day 4, R2 peaked at day 7, and R3 subsets peaked at Day 21 in bone marrow, spleen, liver, lymph nodes and peripheral blood [35]. Immature NK cells (DN and R1) are found in the bone marrow, lymph nodes, and liver and have high rates of proliferation, with DN NK cells displaying the highest rate of homeostatic proliferation [35, 37]. Mature NK cells, characterized by CD11b+ surface markers (R2 and R3 subsets), are found in peripheral sites such as spleen, peripheral blood mononuclear cells (PBMCs), and lung. They have been seen to only undergo proliferation during acute inflammatory states such as infection leading to more NK cells with mature phenotypes. Hayakawa et al. further differentiated the mature subsets by their expression of CD27 and found that CD27+ cells (R2 subsets) had greater effector functions such as increased NK cytotoxicity compared to CD27- (R3 subsets) [36]. Additional increased effector functions in CD27+ cells include enhanced responsiveness to

activating ligand expressed on tumor cells (NKG2D), greater production of IFN-γ when stimulated with IL-12 and IL-18, higher responsiveness to dendritic cells as determined by secretion of IFN-γ, and had lower activation threshold. CD27+ (R2 subsets) were also the dominant fraction in lymph nodes and they expressed CXCR3 allowing active chemotaxis to chemokines such as CXCR3 ligands [36].

Natural Killer Cell Activation by Cytokines

During viral infections, activated innate cells such as dendritic cells and macrophages secrete various cytokines to activate NK cells [31]. Interleukin 15, 18, 12, 2 (IL-15, IL-18, IL-12, IL-2) and Type I IFN are potent cytokines that activate NK cell cytotoxicity, NK cell proliferation, and NK cell secretion of cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), which stimulate antiviral defense mechanisms within the target cells and activates other immune cells [31, 33] (shown in Figure 1). Type I interferons (IFN-α/β) have anti-tumor and anti-viral activities, partially due to its ability to induce activation of NK cells in vivo and in vitro and induce NK cell secretion of inflammatory cytokines IFN-γ and TNF-α [38, 39]. IL-10 is normally known as an anti-inflammatory cytokine, but studies show that IL-10 is an activator of NK cells, increasing their killing ability [40]. After an extended infection, NK cells proliferate and IL-10 production is increased through histone modifications in the IL-10 gene allowing for transcription [41]. Furthermore, IL-15 can induce IL-10 secretion from NK cells and increase NK cytotoxicity *in vitro* [40, 42, 43].

Natural Killer Cell and Aging

As stated above, NK cells are important in fighting viral infections and cancerous growth and impairment in NK cell cytotoxicity can lead to increases in susceptibility to diseases. Research shows that there is an age associated decline in NK cell activity in mice which increases age-

dependent susceptibility to influenza infection [34] and other viral infections [44]. Previous research by Plett et al. found that basal spleen NK cytotoxicity in adult (6 months) and aged (24 months) C57BL/6 mice were comparable although both age groups had significantly lower NK cytotoxicity compared to young mice (2 months) [38]. They used the standard 51Cr-release assay with the mouse lymphoma cell line YAC-1 target cells at 100:1 effector to target cell (E:T) ratio. However, the same group of researchers found that IFN-α/β treatment *in vivo* and *in vitro* increased NK cell activity of 6 month old mice but not 22 months old mice indicating an age-dependent decrease in cytokine-induced NK cell activity [39]. There were no differences in percent of spleen NK cells in lymphocyte in aged and adult mice, although aged mice had significantly higher percentages of CD8+NK1.1+ cells. Their findings suggested that the altered response to IFN-α/β signaling may explain the differences in NK cytotoxicity in aged versus adult mice [39]. Despite higher expression of IFN-α/β receptors, aged mice had impaired IFN-α/β binding compared to adult mice. In addition, IFN-α/β stimulation led to increases in the apoptosis signaling molecule Fas (CD95) expression and apoptosis of NK cells in aged mice.

Nogusa et al. demonstrated that aged mice (22 months) had impaired influenza stimulated increases in NK cytotoxicity compared to young mice (6-8 weeks) using the same assay but with (E:T) ratio of 50:1 [45]. The investigators infected young and aged mice with PR8 influenza A virus and measured NK cell activity in both lung and spleen for three days after infection. The investigators found that young mice had peak NK cell activity day 2 post infection in both lung and spleen, after which NK cell activity decreased in both tissues. However, aged mice had modest increases in NK cell activity after infection, and had 80% decreased activity compared to young mice on day 2 in both the lung and spleen. In addition to the reduced influenza-induced NK cell activity, aged mice had reduced NK cell number in lung, higher viral titer, and greater

weight loss compared to young mice. The investigators found that the response of the aged mice to influenza infection is similar to young mice depleted of its NK cells, highlighting the importance of NK cells during the early response to influenza infection.

Beli et al. infected C56BL/6J male young (6 month) and aged (22 month) mice with the mouse adapted strain of H1N1 influenza A/PR8 strain and determined the reason behind increased susceptibility to flu strains in aging mice [34]. The investigators found that aged mice had significantly lower NK cells (percent and absolute number) in lung and spleen at baseline and after infection. They also determined NK cell activity at baseline and day 2 post-infection. They did this by stimulating isolated spleen and lung lymphocytes with YAC-1 cells, a murine lymphoma cell line, at the 10:1 E:T ratio and incubating with the protein transporter inhibitors brefeldin and monensin. They found that aged mice had similar degranulation compared to young mice, as determined by the CD107a surface mobilization assay, at baseline in both the lung and spleen. However, after 2 days post infection, aged mice had significantly less degranulation compared to young mice in both organs. The secretion of IFN-γ by activated NK cells is important in controlling viral infection. The investigators found that there were no differences in baseline levels of IFN-γ secretion from lung NK cells with YAC-1 stimulation. However, after 2 days post infection, aged mice had increased IFN-γ secretion compared to baseline levels, but it was significantly lower than young mice. In spleen tissue, aged mice had significantly less IFN-γ secretion at both time points measured. In addition, the investigators found that aged mice had more immature (R1-CD27+, CD11b-) NK cells and less terminally mature (R3- CD27-, CD11b+) cells compared to young mice at baseline and 2 days post infection in both lung and spleen tissue. Overall, the authors suggest that aging may increase

susceptibility to influenza infection due to decreases in NK cell number, function, and phenotype.

Significance of Decreased NK Cell Cytotoxicity

NK cells provide early defense against pathogenic organisms during the initial response period while the adaptive immune system is activated, and this is especially important for viral infections. Mice with NK cell depletion had increased susceptibility to herpes virus MCMV [31, 46]. In humans, natural killer cell deficiencies also led to increased susceptibility to infection, particularly herpes viral infection [47]. A prospective cohort study in Japanese participants showed that individuals with low NK cell cytotoxicity had increased risk for developing cancer, which supports the importance of NK cell cytotoxicity in immune surveillance [48]. In addition, a cross sectional study by Ogata et al. found that low NK cell activity was associated with the development of infection and shorter survival due to infection in 108 immunologically normal elderly subjects in nursing homes [49]. Thus the finding that presence of unmetabolized folic acid in plasma is associated with decreased NK cytotoxicity in older women may have negative implications for cancer development and susceptibility to viral infection in older populations.

Potential Mechanisms behind the Effects of Excess Folic Acid on NK Cell Cytotoxicity

There are many potential mechanisms behind the decreased NK cell cytotoxicity with excess consumption of folic acid. One possible mechanism is through the presence of unmetabolized folic acid when folic acid is consumed in excess of the body's ability to reduce it to DHF and 5MTHF. Folic acid can be oxidized and broken down to yield 6-formyl pterine (6FP) and aminobenzoylglutamate (PABAGlu) (Figure 2).

Studies suggest that the breakdown product, 6-formyl pterin (6FP), is a bioactive compound that can modify immune function [50-52]. 6FP incubated with human peripheral

blood leukocytes induced intracellular generation of hydrogen peroxide (H_2O_2) by transferring an electron from NADH to oxygen [53]. Human T cells incubated with 6FP had increased internal reactive oxygen species (ROS), decreased cell proliferation, and impaired secretion of cytokines IFN-γ and IL-2 through suppressed NF-κB-dependent transcription when stimulated with mitogenic lectin phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) [50]. A study by the same group showed that 6-FP inhibited nitric oxide synthesis in the murine macrophage cell line RAW 264.7 when stimulated with LPS and IFN-γ, suggesting an inhibition of inflammatory signaling by activated macrophages [52]. Activated macrophages can secrete cytokines that are known to activate NK cells [31], thus 6-FP may be able to inhibit NK cell activity through inhibition of activated macrophages.

Both younger and older postmenopausal women had similar concentrations of unmetabolized folic acid in plasma. However, only the older postmenopausal women (ages 60-75) had decreased NK cytotoxicity with increasing unmetabolized folic acid [1]. A hypothesis is that there may be greater breakdown of folic acid in the elderly, leading to increased 6FP, which directly modifies the functions of immune cells. Pregnant women have increased breakdown of folic acid as determined by the concentration of its breakdown product, PABAglu, in urine [54], and this might be due to increased oxidative stress during pregnancy. Similarly, oxidative stress increases with age, which may lead to increased breakdown of folic acid to 6FP.

Another potential mechanism by which excess folic acid is altering NK activity is through changing methylation of genes related to NK activity. It has previously been shown that feeding rodents with a high folic acid diet altered global methylation [55], and studies report that IL-10, a cytokine that activates NK activity, is regulated through methylation of its promoter region [56, 57]. Excess folic acid may alter methylation reactions by either providing more

substrate for methylation reactions, or it may be inhibiting certain reactions in the one carbon metabolism pathway, as suggested by Rowe and Lewis et al. [58], and this could lead to a build-up of homocysteine.

Influenza Infection

Morbidity and mortality during influenza epidemics are particularly high among older people [59, 60], with 90% of all deaths due to the flu occur in those >65 years old [61]. In addition, complications of the influenza infection in the elderly such as hospitalization can lead to muscle loss during periods of inactivity and eventually to frailty [62]. Frailty is linked to loss of independence and increased mortality [63].

Viral infection results in acute phase responses such as lethargy, fever, and weight loss [64]. Cytokines such as IL-1, IL-6, TNF-α, and IFNs are known to increase during viral infection and can cause both fever and weight loss [64]. Studies have shown an age-related loss of vaccine-mediated protection against influenza, with one randomized double-blind placebo-controlled trial conducted in participants >60 years old in the Netherland finding that vaccination only reduced the incidence of influenza by 50% in elderly [62, 65]. A Cochrane review indicate that effectiveness of vaccination against the influenza may be as low as 30% in the elderly, which is much lower than efficacy in healthy adults [66]. This reduced efficacy of vaccination in healthy elderly is attributed to impaired humoral and adaptive immune system[62]. In the absence of protection from vaccination, the immune system is important for controlling infection and preventing damage. The innate immune system plays an important role in controlling severity of influenza infection through NK cell surveillance and cytotoxicity in the early stage of infection.

If NK cell function is decreased in older populations with very high consumption of folic acid, it may increase their susceptibility to infection. This hypothesis should first be tested in animal models before determining whether there is a similar effect in human beings. If this is the case, there should be a public health intervention to decrease consumption of folic acid above the RDA, particularly through supplements. In Chapter III of this thesis, we'll determine if excess folic acid intake in an aged mouse model will increase severity and duration of influenza infection.

Folate and Vitamin B-12 Interaction

Vitamin B-12 or cobalamin is a water soluble vitamin that serves as a coenzyme in two reactions: the conversion of methylmalonyl-CoA to succinyle-CoA by the enzyme methylmalonyl-CoA mutase; and the re-methylation of homocysteine to methionine by the enzyme methionine synthase. Methionine is used in the synthesis of S-adenosine methionine (SAM), which is needed for the maintenance of the nervous system through methylation of proteins, nucleic acid, myelin, neurotransmitters, and membrane phospholipids (Figure 3). A deficiency in vitamin B-12 may lead to development of cognitive impairment, depression, combined degeneration of the spinal cord, peripheral neuropathy, anemia, and other diseases [67]. Peripheral neuropathy is a disease characterized by peripheral nerve demyelination and damage. According to the National Institute of Neurological Disorders and Stroke, peripheral neuropathy affects an estimated 20 million people in the United States [68], with one study reporting a 31% prevalence in participants ≥65 years old [69].

The recommended dietary allowance (RDA) for vitamin B-12 is 2.4 μg/d for adults. Elderly are at increased risk for vitamin B-12 deficiency due to malabsorption or impaired production of intrinsic factor (IF) which is needed to bind and absorb vitamin B-12 in food and

for increased use of antacids. The prevalence of vitamin B-12 deficiency in those older than 60 years old is 6% and 20% of individuals in this age group have marginal vitamin B-12 status in the United States [70-72]. People with marginal vitamin B-12 status are still susceptible to the clinical symptoms of vitamin B-12 deficiency [73].

After ingestion, vitamin B-12 in foods or supplements binds to IF in the stomach and the B-12/IF complex is taken up by enterocytes in the ileum. Vitamin B-12 is then transferred to the binding protein transcobalamin 2 (TCN2) and the complex is referred to as holotranscobalamin (holo-TCN) (Figure 3). Although vitamin B-12 may be bound to other transport proteins and only a third of vitamin B-12 is bound to TCN2, only TCN2 facilitates the uptake of vitamin B-12 into tissues via endocytosis [74].

Certain polymorphisms in this protein may alter the transporter's ability to deliver vitamin B-12 to cells. This may result in impaired intracellular delivery of vitamin B-12 and increased plasma levels of substrates from vitamin B-12 dependent reactions such as methylmalonic acid (MMA) and homocysteine. Previous studies have shown that the TCN2 C776G polymorphism (rs1801198), which leads to the replacement of proline 259 with arginine, may affect TCN2 function [75-85]. This occurrence of the polymorphism in the population is common with the percent of people with the GG polymorphism ranging from 11-28% [76-78, 81, 83-86]. Structural data and crystal structure of the polymorphism showed that the polymorphism is not located in a key region and should not influence binding ability or stability of the protein [87, 88]. However, one study suggests that the GG polymorphism impairs the binding of TCN2 to vitamin B-12 [75] and another showed expression of the protein is reduced in vitro [89]. The GG polymorphism is associated with decreased holo-TCN [75, 77, 81, 84, 85] and increased concentrations of plasma homocysteine [76, 79, 83]. It is controversial whether

the GG TCN2 polymorphism is associated with decreased total plasma vitamin B-12 since the TCN2 polymorphism should affect the transport protein's ability to bind and transfer the vitamin to peripheral cells and not absorption of vitamin B-12 [76-78, 81, 82, 85]. However, one study showed decreased plasma vitamin B-12 with the GG TCN2 polymorphism [83]. Although there have been many studies that have looked at the TCN2 C776G polymorphism and biochemical markers of one carbon metabolism, no known studies have determined if the TCN2 polymorphism is associated with clinical outcomes associated with vitamin B-12 deficiency in the elderly in those with normal intake of vitamin B-12.

In addition, studies from our lab showed that high folate consumption in conjunction with vitamin B-12 deficiency or marginal status led to increased prevalence of cognitive impairment in the elderly compared to those with normal folate consumption [29, 90-92]. Morris et al. found that low vitamin B-12 status (plasma concentrations <148 pmol/L) was associated with increased risk of anemia, macrocytosis, and cognitive impairment as determined by the Digit Symbol-Coding score in a population of older participants (age ≥ 60 years) in the 1999-2002 United States National Health and Nutritional Examination Survey (NHANES) [29]. Interestingly, those with low vitamin B-12 status and high serum folate concentrations (>59 nmol/L) had higher risk of anemia and cognitive impairment than those with low vitamin B-12 status and normal serum folate. In addition, the authors found that two functional indicators of vitamin B-12 status, total homocysteine (tHcy) and methylmalonic acid (MMA), rose with increasing serum plasma folate (>20 nmol/L) when subjects had low vitamin B-12 status (<148 pmol/L) [91]. Both studies showed that with higher vitamin B-12 status (>148 pmol/L), high folate concentrations was protective against anemia, cognitive decline, decreased tHcy, and MMA [29, 91]. These findings

suggest that high folate status with low vitamin B-12 status worsened both clinical and biochemical symptoms of vitamin B-12 deficiency.

The previous studies reported were cross sectional and thus more evidence is needed to support their findings. An 8 year prospective study examining participants in the Framingham Heart Study found that cohort members in the lowest two plasma vitamin B-12 quintile category (187-256.8 pmol/L) had the fastest cognitive decline as measured by the Mini-Mental State Examination (MMSE) [90]. In addition to this finding, the authors showed that cohort members with this range of plasma vitamin B-12 in conjunction with high plasma folate had an even faster cognitive decline. The mechanism is not yet known, but this issue has public health implications since people older than 50 years have a mean intake of 900 μg/d of folate in the United States [93]. This is greater than 2x the RDA for folate, mostly due to the high prevalence of people in this age group who consume supplements and multivitamins in addition to eating foods fortified with folate. If the TCN2 C776G polymorphism leads to a functional vitamin B-12 deficiency despite sufficient intake, then presence of this polymorphism may lead to increased risk for B-12 clinical symptoms in those who have high intakes of folate.

In chapter IV and appendix Part II, we used cross sectional data from the Nutrition, Aging, and Memory in Elders (NAME) Study to determine the association of the TCN2 C776G polymorphism with plasma concentrations of vitamin B-12 and homocysteine, and clinical symptoms of vitamin B-12 such as cognitive impairment and peripheral neuropathy. We also determined if these relationships were modified by a high folate intake in homebound elderly individuals older than 60 years. We hypothesized that those with the GG variant genotype will have lower concentrations of vitamin B-12, higher concentrations of plasma homocysteine, increased cognitive impairment, and greater odds of peripheral neuropathy compared to the

dominant CC genotype. In addition, we hypothesized that high folate intake would exacerbate these associations.

Conclusion

In this review, we've summarized thus far the impact of excess folic acid intake on chronic diseases, immune function, and vitamin B-12 deficiency. We have also suggested some potential mechanisms behind the effects of excess folic acid consumption based on existing evidence in the literature. More importantly, we've identified gaps in the research regarding the causal effect of excess folic acid on immune function and if excess folic acid can make symptoms of vitamin B-12 deficiency worse in a population replete with vitamin B-12.

Further research is needed with respect to: 1) determine if there is a causal relationship between excess folic acid intake and decreased natural killer cell cytotoxicity; 2) if excess folic acid intake will lead to increased susceptibility to infections; and 3) if TCN2 polymorphism is associated with clinical symptoms of vitamin B-12 deficiency despite sufficient intake, and if excess folic acid intake would exacerbate the association between the polymorphism and clinical symptoms of vitamin B-12 deficiency.

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Figure Legends

Figure 1. Regulation of NK cell activity. During infections, other innate cells such as macrophages and dendritic cells (DC) can secrete cytokines such as IL-15, IL-18, Type I IFN, IL-12, and IL-2 which can stimulate NK cells to mature and increase NK cytotoxicity and NK secretion of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). IL-10, secreted by NK cells themselves or peripheral immune cells, is also a potent activator of NK cells.

Figure 2. Folic Acid breakdown to 6-Formyl pterine (6FP) and ρ-aminobenzoylglutamate (PABAGlu). Under conditions of oxidative stress, folic acid breaks down to 6-formylpterine (6FP) and p-aminobenzoylglutamate (PABAGlu). On the other hand, 5-methyl-THF breaks down into 4-α-hydroxy-5methyl-THF.

Figure 3. Metabolism of vitamin B-12. Vitamin B-12 is bound to intrinsic factor or IF in the stomach. In the ileum, the vitamin B-12/IF complex is taken up by enterocytes, where vitamin B-12 is then transferred to the binding protein transcobalamin 2 or TC2. When vitamin B-12 is bound to TC2, this complex is now called Holo-TC. Only 30% of plasma vitamin B-12 is bound to TC2, while the rest of vitamin B-12 is bound to other binding proteins. However, Holo-TC is considered the "active fraction" because it facilitates cellular uptake. Vitamin B-12 is then used in the following reactions as a coenzyme in peripheral cells: the conversion of L-methylmalonyl-CoA to succinyl-CoA through the enzyme L-methylmalonyl-CoA mutase. Succinyl-CoA enters the Krebs cycle and is an essential reaction in fat and protein metabolism; and methionine synthase catalyzes the conversion of homocysteine to methionine, which is required for the formation of S-adenosylmethionine, a universal methyl donor. This is important for methylation of DNA, RNA, proteins, lipids and is important for the integrity of myelin sheath and neurotransmitter formation.

Figure 1.

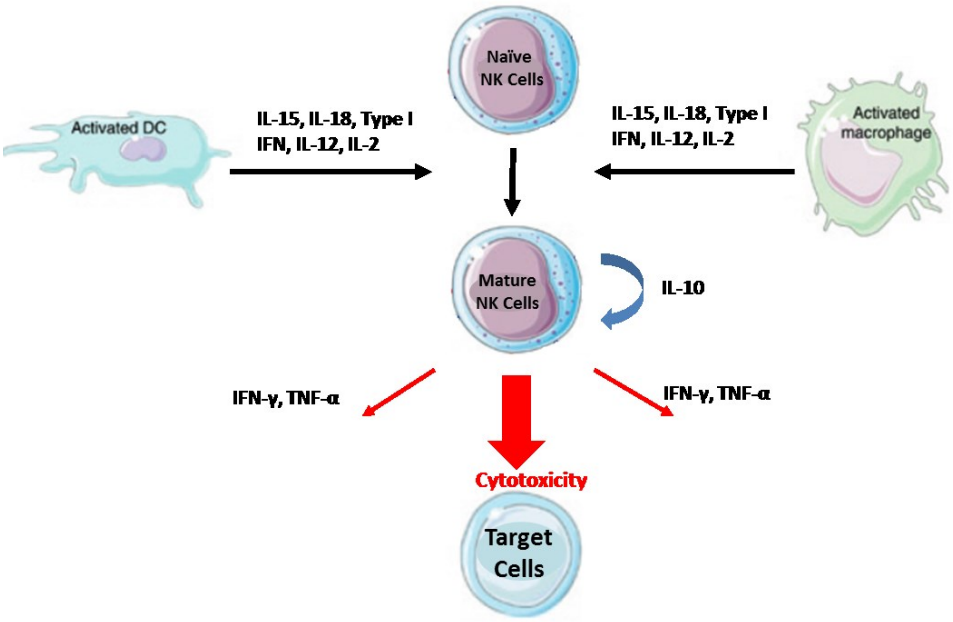

Naïve
NK Cells
Activated DC
IL-15, IL-18, Type I
IFN, IL-12, IL-2
IL-15, IL-18, Type I
IFN, IL-12, IL-2
Activated
macrophage
Mature
NK Cells
IL-10
IFN-γ, TNF-α
IFN-γ, TNF-α
Cytotoxicity
Target
Cells

Figure 2.

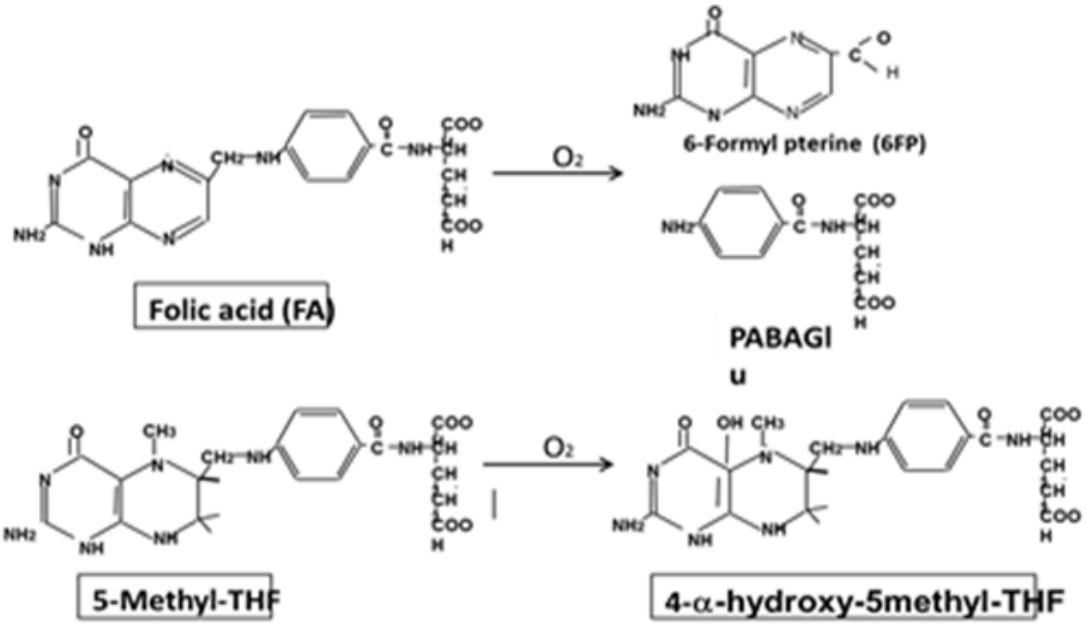
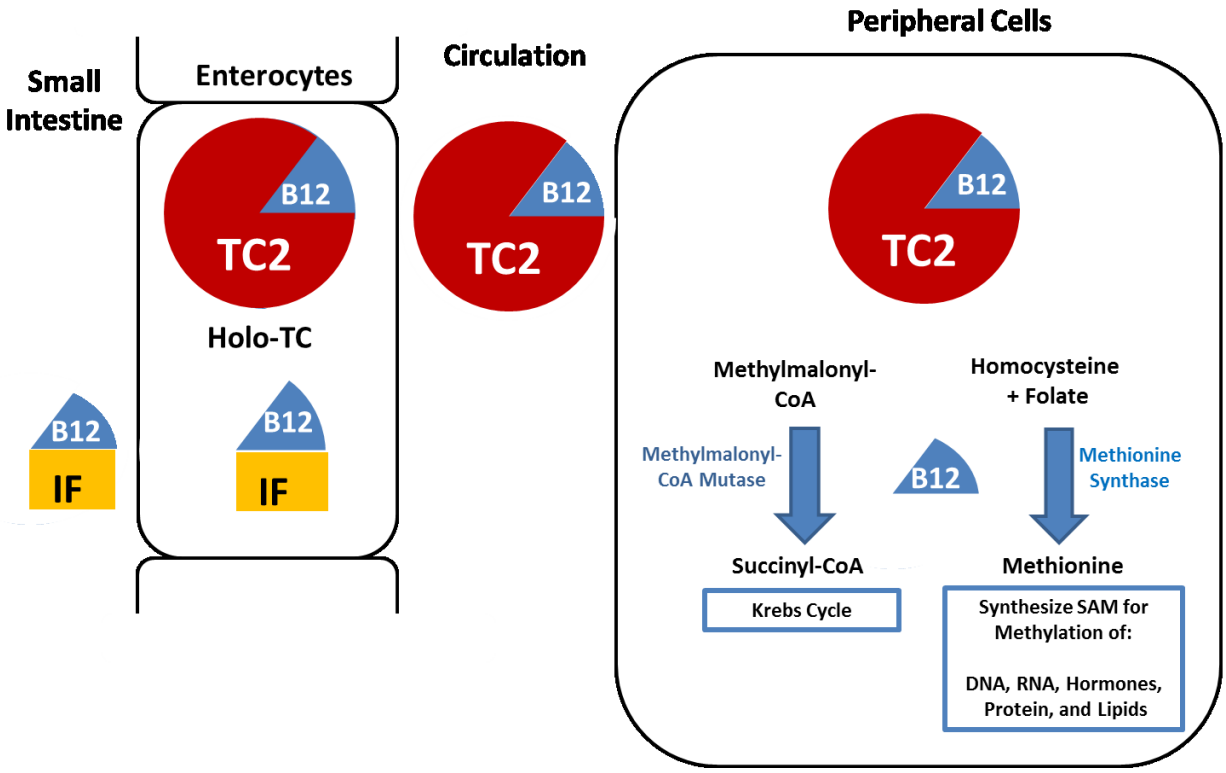

6-Formyl pterine (6FP)
Folic acid (FA)
PABAGlu
5-Methyl-THF
4-α-hydroxy-5methyl-THF

Figure 3.


Small Intestine
Enterocytes
Circulation
Peripheral Cells
B12
TC2
Holo-TC
B12
IF
B12
IF
B12
TC2
B12
TC2
Methylmalonyl-CoA
Homocysteine + Folate
Methylmalonyl-CoA Mutase
B12
Methionine Synthase
Succinyl-CoA
Methionine
Krebs Cycle
Synthesize SAM for Methylation of:
DNA, RNA, Hormones, Protein, and Lipids

Chapter II

High Folic Acid Intake Reduces Natural Killer Cell Cytotoxicity in Aged Mice

High Folic Acid Intake Reduces Natural Killer Cell Cytotoxicity in Aged Mice☆

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☆Conflict of interest: The authors declare no conflict of interest

Running title: High folic acid diet & natural killer cell cytotoxicity

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Keywords: Folic acid, folate, natural killer cell cytotoxicity, interleukin-10.

Abstract

Presence of unmetabolized folic acid in plasma, which is indicative of folic acid intake beyond the metabolic capacity of the body, is associated with reduced natural killer (NK) cell cytotoxicity in post-menopausal women ≥ 50 years. NK cells are cytotoxic lymphocytes that are part of the innate immune system critical for surveillance and defense against virus-infected and cancer cells. We determined if a high folic acid diet can result in reduced NK cell cytotoxicity in an aged mouse model. Female C57BL/6 mice (16-month-old) were fed an AIN-93M diet with the recommended daily allowance (1x RDA, control) or 20x RDA (high) folic acid for 3 months. NK cytotoxicity was lower in splenocytes from mice fed a high folic acid diet when compared to mice on control diet ($P<0.04$). The lower NK cell cytotoxicity in high folic acid fed mice could be due to their lower mature cytotoxic /naïve NK cell ratio ($P=0.03$) when compared to the control mice. Splenocytes from mice on high folic acid diet produced less interleukin (IL)-10 when stimulated with lipopolysaccharide ($P<0.05$). The difference in NK cell cytotoxicity between dietary groups was abolished when the splenocytes were supplemented with exogenous IL-10 prior to assessment of the NK cytotoxicity, suggesting that the reduced NK cell cytotoxicity of the high folic acid group was at least partially due to reduced IL-10 production. This study demonstrates a causal relationship between high folic acid intake and reduced NK cell cytotoxicity and provides some insights into the potential mechanisms behind this relationship.

Keywords: Folic acid, folate, natural killer cell cytotoxicity, immune function; interleukin-10.

1. Introduction

Recent human and animal studies have shown that intake of excess folic acid has been linked to a variety of negative health outcomes, including increased risk for cancer, insulin resistance, allergies, behavior modification and birth defects [1-4]. Folic acid, the synthetic form of the vitamin folate (B9) is used in supplements and fortified cereal products due to its stability when compared to other forms of folate. The recommended daily allowance (RDA) for folate is 400 µg dietary folate equivalents, which is equal to 400 µg natural folate or 240 µg folic acid [5, 6]. Approximately 35% of the US population consumes folic acid containing dietary supplements and 5% exceed the tolerable upper intake level of 1000 µg/d for folic acid [7]. Many B-vitamin supplementation trials focusing on age-related chronic illnesses use doses of folic acid at 5000 µg/d or higher [8-11] which is approximately 20 fold the RDA or more. Women who are at risk for neural tube defect pregnancies are recommended to take 4000-5000 µg folic acid/d [12-14]. Folic acid must be reduced by dihydrofolate reductase (DHFR) to tetrahydrofolate before it can enter the metabolic pathway. In humans, DHFR is a slow enzyme with poor affinity for folic acid [15]. In addition, genetic variations in DHFR also influence the ability of an individual to metabolize folic acid [16]. We have previously shown that the presence of unmetabolized folic acid in plasma, indicative of folic acid intake beyond the metabolic capacity of the body, is associated with reduced natural killer (NK) cell cytotoxicity in postmenopausal women aged 50-75 years [17]. NK cells are cytotoxic lymphocytes that are part of the innate immune system and are important for surveillance and defense against virus-infected and cancer cells. They bind to target cells and perforate the cell membrane by secreting pore-forming protein perforin and trigger apoptosis by secreting granzymes [18]. Low NK cytotoxicity is associated with increased risk for cancer in humans [19]. Hence it is important to determine if there is a causal relationship between high folic acid intake and NK cytotoxicity.

2. Materials and Methods

2.1. Animals and diets.

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and conducted according to the Guide for the Care and Use of Laboratory Animals (1996). Sixteen-month-old female C57BL/6 mice were purchased from National Institute of Aging colonies at Charles River Breeding Laboratories (Wilmington, MA). We sought to determine the effect of high folic acid intake on immune function in an aged mouse model since the initial observation was reported in a study of women 50 years and older [17]. The mice were housed on a 12 hour light/dark cycle and provided free access to the diets throughout the experiment. Twelve mice per diet group were maintained on an AIN-93M [20] based diet (Harlan-Teklad, Madison, WI) with the American Institute of Nutrition recommended daily allowance of 2 mg/kg diet folic acid (control diet, 1x RDA) or 40 mg/kg diet folic acid (high folic acid diet, 20x RDA) for 3 months. The dosage of folic acid in the high folic acid diet was determined based on a previous study that investigated the effect of high folic acid diet in mice [21]. In addition, equivalent dosage of folic acid is used in many intervention trials of age-related chronic illnesses [8, 9].

2.2. Spleen cell isolation.

Mice were euthanized by CO_2 asphyxiation followed by exsanguination through cardiac puncture, and blood was used for plasma isolation. Spleen was removed aseptically and single-cell suspensions were prepared as previously described [22]. Spleens were placed in sterile RPMI 1640 medium (Lonza, MA) which was supplemented with 5% heat inactivated fetal bovine serum, 25 mmol/L HEPES, 2 mmol/L glutamine, 100 kU/L penicillin, and 100 mg/L streptomycin (Life Technologies, CA) and disrupted between two sterile frosted glass slides. The cells were collected by centrifugation at 300G for 10 min and resuspended in red blood cell lysis buffer (Sigma, MO) to lyse red blood cells. After washing with phosphate buffered saline and

removing cell debris by filtering through 40 μM nylon cell strainer (BD Pharmingen), splenocytes were counted using an Accuri C6 flow cytometer (BD Accuri Cytometers, MI) and resuspended in appropriate media for the assays.

2.3. Preparation of target cells.

YAC-1 cells, a murine T- lymphoma cell line sensitive to NK cell killing, were grown in complete RPMI 1640 medium prepared as above and maintained at 37°C in 5% CO_2. For the 5 days before the assay, cells were subcultured every 24 h to ensure that they were in the log phase. The cells used for the assay were stained with 10 nM of carboxyfluorescein succinimidyl ester (CFSE, eBioscience, CA) for 10 minutes at room temperature in the dark, washed twice in phosphate buffered saline with 2% heat-inactivated fetal bovine serum and resuspended to a final concentration of 10^6 cells/mL in RPMI 1640 medium.

2.4. NK cytotoxicity assay.

The flow cytometric assay described by McGinnes et al [23] was used with modifications as described by Cao et al. [24]. All reagents were purchased from eBioscience. Splenocytes (10^7/tube) were stained with allophycocyanin (APC)-conjugated anti-CD3 antibody and phycoerythrin (PE)-conjugated anti-NK1.1 antibody to identify NK cells ($CD3^-$ $NK1.1^+$). Stained splenocytes were then incubated with CFSE-labelled YAC-1 cells (target cells) at effector-to-target (E:T) cell ratios of 100:1, 50:1, 25:1, and 12.5:1 for 3 h at 37C with 5% CO_2. After the incubation was complete, 7-amino-actinomycin D (7-AAD) was added to each tube to a final concentration of 1.11 μg/mL. Dead YAC-1 cells, which were identified as 7-AAD and CFSE double positive cells, were counted using an Accuri C6 flow cytometer and acquired data were analyzed with FlowJo 7.6 software (Treestar Inc., OR). CFSE stained YAC-1 cells incubated in the absence of splenocytes were used to determine spontaneously dead target cells. Percent specific target cell death (cytotoxicity) was then expressed as: 100 x {[dead YAC-1 Cells

(%) – spontaneously dead YAC-1 Cells (%)]/ [100 – spontaneously dead YAC-1 target cells (%)]}.

2.5. Immunophenotyping.

Splenocyte populations were determined using flow cytometry. Splenocytes were blocked using anti-CD16/32 antibodies and then stained using the following fluorochrome-conjugated antibodies: PE or APC-anti-CD3, APC-anti-CD4, fluorescein isothiocyanate (FITC) anti-CD8 and PE-anti-NK1.1 (eBioscience), to identify all T cells ($CD3^+$), T-helper cells ($CD3^+$ $CD4^+$), cytotoxic T cells ($CD3^+$ $CD8^+$), NK cells ($CD3^-$ $NK1.1^+$) and natural killer T (NKT) cells which have the properties of both T cells and NK cells ($CD3^+$ $NK1.1^+$). The stained cells were then analyzed using Accuri C6 flow cytometer and acquired data analyzed with FlowJo 7.6 to determine the immune cell phenotype.

Natural killer cell subsets were identified as previously described [25] using the following fluorochrome conjugated antibodies: APC-anti-CD3, PE-anti-NK1.1, FITC-anti-CD27 and PerCP-Cy5.5-anti-CD11b. All staining reactions included isotype controls to detect non-specific background signals. The antibodies and the respective isotype controls were purchased from eBioscience. The stained cells were analyzed using an Accuri C6 flow cytometer and the data were analyzed with FlowJo 7.6. Since a small portion of spleen was removed for folate analysis and the remaining spleen was immediately aseptically transferred to cell culture medium without weighing, the absolute numbers of the various cell types in spleen were not determined. Populations of various cell types were instead determined as percentage of total splenocytes.

2.6. Cytokine production.

Splenocytes in complete RPMI 1640 medium were stimulated in separate reactions with bacterial lipopolysaccharide (LPS, 1μg/mL) for 24 h, concanavalin A (Con A) (1.5μg/mL), or anti-CD28 antibody (1 μg/mL) and anti-CD3 antibody (5μg/mL) coated on cell culture plates for

48 hours . At the end of the incubation, cell-free supernatants were collected and stored at -80°C until analysis. The concentration of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and interferon (IFN)-γ in the supernatants from LPS stimulated cells, and the concentration of IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF- α in the supernatants of Con A or anti-CD3and anti-CD28 stimulated cells were determined using the mouse cytometric bead array kit according to the manufacturer's instructions (CBA; BD Biosciences, CA). The fluorescence signals associated with cytokine-bead complex were acquired using Accuri C6 flow cytometer, and data were analyzed using FCAP Array™ Software 3.0.1 (CBA; BD Biosciences).

2.7. Stimulation of NK cytotoxicity by IL-10.

Splenocytes ($6x10^6$/well) were incubated with recombinant mouse IL-10 (eBioscience) at a final concentration of 15 ng/mL for 15 hours before measuring NK cytotoxicity as described above for the E:T ratios 25:1 and 12.5:1.

2.8. Folate analysis.

Total folate from spleen was determined using a microbial assay with *Lactobacillus casei* [26]. Protein concentration of the spleen extract used for folate assay was determined by Bradford method [27] using Bio-Rad protein assay reagent (Bio-Rad, CA). Folate forms in non-fasting plasma samples were analyzed by HPLC-affinity chromatography with electrochemical detection [28].

2.9. Statistical analyses.

Results are expressed as means ± SEM. One way ANOVA was used for the effect of diet on NK cell cytotoxicity adjusted for day of experiment. Student's t-test was used for all other continuous outcomes. Significance was determined at $P<0.05$. Statistical analysis was performed using SAS 9.3 (NC).

3. Results

3.1. Effect of high folic acid diet on tissue and plasma folate content.

Animals on both control and high folic acid diets gained weight during the 3 months on experimental diets, but there was no difference between the two diet groups in weight gain (data not shown). Analysis of non-fasting plasma showed that mice on high folic acid diet had significantly higher concentration of unmetabolized folic acid, methyl tetrahydrofolate and formylated tetrahydrofolate when compared to mice on the control diet (Fig. 1A). Since the NK cell cytotoxicity was determined using splenocytes, we also determined the folate concentration of spleen. Mice fed a high folic acid diet had a higher concentration of total folate in spleen when compared to those on the control diet (18.21 ± 0.76 vs 12.38 ± 0.96 ng folate /mg protein, $P<0.01$, Fig. 1B).

3.2. Effect of high folic acid diet on NK cell cytotoxicity.

The mean NK cell cytotoxicity of mice fed a high folic acid diet was significantly lower than that of the mice fed the control diet at effector to target cell ratios of 25:1 ($P=0.03$) and 12.5:1 ($P=0.04$) by 14% and 23% respectively (Fig. 2). We determined if the difference in NK cell cytotoxicity of the mice on the two diets was due to differences in the percentage of NK cells. Since the NK cell cytotoxicity assay measures the activity of both NK and NKT cells, we also measured the percentage of NKT cells in the spleen. While the percentage of NK cells in the spleen was lower in the high folic acid group compared to the control group, the difference was not statistically significant ($P=0.15$) (Fig. 3). The percentage of NKT cells in spleen was similar in both diet groups (Fig. 3). The percentages of T helper cells and cytotoxic T-cells in total spleen cells were also similar in both diet groups (Supplementary Fig. 1).

3.3. Effect of high folic acid diet on NK cell subsets

We determined the population of NK subsets in both diet groups based on the presence of the surface markers CD11b and CD27 [29]. Double negative (DN, $CD11b^-$ $CD27^-$) cells are NK cell precursors without effector function, $CD11b^-$ $CD27^+$ (R1 subset) are naïve and have reduced effector functions, $CD11b^+$ $CD27^+$ (R2 subset) are considered mature NK cells capable of killing target cells and secreting cytokines, and $CD11b^+$ $CD27^-$ (R3 subset) are terminally differentiated, mature NK cells that predominantly secretes cytokines [25, 29-31]. In mice fed a high folic acid diet there was a trend for a lower percentage of mature R2 NK cells (P=0.08) and higher percentage of naïve R1 cells (P=0.08) when compared to the control group (Fig. 4A). The percentage of DN and R3 NK cells was similar in both diet groups (Fig. 4A). The ratio of R2 to R1 NK cells (P =0.03), but not that of R3 to R1 was significantly lower in the high folic acid fed mice when compared to those on the control diet (Fig. 4B).

3.4. Effect of high folic acid diet on cytokine production by splenocytes

Since the percentage of NK cells and activation are dependent on cytokines we determined the production of cytokines (IL-10, IFN-γ, IL-6, TNF-α) and chemokine (MCP-1) by splenocytes after LPS stimulation. LPS stimulated a wide array of cell types in the splenocytes. The production of IL-10 by splenocytes from the animals in the high folic acid group were lower than that of the control group (P<0.05) (Fig. 5). The production of other cytokines was not different between the two diet groups. To determine if the low IL-10 production contributes to the reduced NK cell cytotoxicity in the high folic acid fed mice we stimulated the splenocytes with recombinant IL-10 prior to the NK cell cytotoxicity assay. After stimulation with IL-10, the NK cell cytotoxicity of the high folic acid group was similar to that of the control group (Fig. 6). When only the T cells among the splenocytes were specifically activated with Con A or antibodies against CD3 and CD28, there was no difference in the production of cytokines IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF- α between the 2 diet groups (Supplementary Fig. 2A and 2B).

4. Discussion

In this study, we established a causal effect of high folic acid intake on lower NK cell cytotoxicity in mice. Intake of a high folic acid diet resulted in up to 23% lower NK cell cytotoxicity in splenocytes of aged mice when compared to consumption of recommended dose of folic acid ($P < 0.04$) (Fig. 2). This supports our earlier finding that folic acid intake in excess of the metabolic capacity of the body, as indicated by unmetabolized folic acid in plasma, is associated with lower NK cell cytotoxicity in postmenopausal women [17]. In aged mice, a high folic acid diet increased the concentration of total folate in spleen tissue, as well as the concentrations of unmetabolized folic acid, methyl tetrahydrofolate and formyl tetrahydrofolate in plasma when compared to control diet (Fig. 1). We measured the plasma folate concentrations under non-fasting rather than fasting conditions to determine the extent to which the mice are exposed to the various folate forms after consuming the experimental diet. The concentrations of unmetabolized folic acid in mice fed a high folic acid diet were comparable to what was observed in post-prandial serum of human subjects after consumption of 1000 μg of folic acid from bread over a period of 6 hours in 200 μg doses [32].

We further explored the possible mechanisms underlying the effect of high folic acid diet on NK cytotoxicity. Several factors determine the cytotoxic activity of NK cells including the percentage of NK cells, the ratio of mature to naïve NK cells and regulatory cytokines. We found no significant difference in the percentage of NK cells between diet groups, but we noted a significant reduction in the ratio of mature cytotoxic to naïve NK cells in mice fed a high folic acid diet compared to the control diet ($P<0.03$) (Fig. 3). Thus, the reduced NK cytotoxicity in mice fed the high folic acid diet could be due to impaired maturation of NK cells. The sequence of maturation of NK cells in mice is as follows: DN>R1>R2>R3 [31]. DN cells have a very immature phenotype with no effector function. The difference between the diets were observed in R1 and R2 but not R3 cells, probably because R3 is the end-stage of NK cell maturation, and the experimental duration was too short to observe a difference in R3 cells.

NK cytotoxicity is governed by various cytokines, and in addition, NK cells themselves produce cytokines to regulate inflammation and infection [33-35]. The lower IL-10 production by the mice fed the high folic acid diet may also be involved in their lower NK cytotoxicity (Fig. 5). IL-10 is a pleiotropic cytokine which has immunosuppressive functions but at the same time increases cytotoxicity of NK cells [36, 37]. A role for IL-10 in the lower NK cytotoxicity of the high folic acid fed mice is suggested by the fact that, the difference in NK cell cytotoxicity between the dietary groups was abolished when the splenocytes were stimulated by exogenous IL-10 prior to the NK cell assay (Fig. 6).

The mechanism by which a high folic acid diet affects the maturation of NK cells or IL-10 production is not known at present. Based on published research, we speculate that excess folic acid could regulate IL-10 expression via epigenetic mechanisms. IL-10 expression is epigenetically regulated by DNA and histone modifications and hypermethylation of the IL-10 promoter region reduces its expression [38]. In a placebo-controlled folic acid supplementation trial with a dose of 1000 μg/day, higher red cell folate concentration was associated with hypermethylation of promoters in normal colonic mucosa [39]. Based on these evidence, it is possible that a high folic acid diet can potentially influence IL-10 production via its effect on DNA methylation. Further studies have to be conducted to determine if the high folic acid results in hypermethylation of IL-10 promoter region.

Adequate folate nutrition is important for NK cell cytotoxicity and immune response [40, 41]. But high folic acid intake, mostly from consumption of supplements and heavily fortified foods can have negative outcomes. In recent years, many studies have reported association of high concentration of folate in plasma and multivitamin use with increased risk for breast cancer in women [42-44]. A study in rats using varying folic acid content in diets under vitamin B_{12} deficient conditions did not show any effect of supplemental folic acid on NK cytotoxicity [45] possibly due to the short (1 month) duration of the experimental diet and the confounding effect of vitamin B_{12} deficiency. Our study has provided the first evidence for linking excessive folic

acid intake to impaired NK cell function. This observation is of major public health concern since the function of NK cells is immune surveillance against cancerous and pathogen infected cells. Low NK cytotoxicity has been associated with increased risk for cancer in a human study that followed-up the subjects for 11 years [19]. In this study, the NK cell cytotoxicity of the subjects were categorized as low, <42%; medium, 43-58%; and high, >58% for men; and low, <34%; medium, 35-51%; and high, >51% for women, and their risk for development of cancer determined. Men and women in the low NK cell cytotoxicity group were at higher risk for development of cancer when compared to those in the medium or high groups [19]. Hence, reduction of NK cell cytotoxicity due to high folic acid intake may increase the susceptibility to cancer and viral infections especially in the elderly who are already at increased risk for these diseases.

In summary, our data show that high folic acid intake reduces NK cell cytotoxicity in old mice and this is possibly due to impairment of NK cell maturation. Our data also suggest a role for IL-10 in the observed reduction of NK cell cytotoxicity. Additional studies are needed to further determine the mechanism behind the effect of excess folic acid intake on NK cell cytotoxicity and establish its relevance to health outcomes in humans.

Acknowledgements

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Figure Legends

Fig. 1. Effect of a high folic acid diet on folate concentration in plasma and spleen of aged mice. (A) Folate forms in plasma as measured by HPLC. FA, unmetabolized folic acid; FF, formylated folates; MTHF, methyl tetrahydrofolate. (B) Total folate concentration in spleen as measured by microbial assay using *Lactobacillus casei*. Values are means ±SEM, *n*=10-11/group. * P<0.01.

Fig. 2. Effect of a high folic acid diet on NK cell cytotoxicity in aged mice. Splenocytes were isolated and incubated with YAC-1 target cells at 100:1, 50:1, 25:1, and 12.5:1 ratio of effector to target cells. Dead target cells (YAC-1 cells, CFSE+, 7-AAD+) were quantified by flow cytometry. Values are means ±SEM, *n*=10-11/group. * P<0.05

Fig. 3. Effect of a high folic acid diet on percent NK cells in splenocytes in aged mice. Percent of NK ($CD3^-$ $NK1.1^+$) or NKT ($CD3^+$ $NK1.1^+$) cells in total splenocytes quantified by flow cytometry. Values are means ±SEM, *n*=10-11/group. * P<0.05

Fig. 4. Effect of a high folic acid diet on NK cell subsets in splenocytes in aged mice. Splenocytes were isolated and stained with fluorescent dyes conjugated to anti-CD11b, CD27, and NK1.1 antibodies and quantified by flow cytometry. (A) Representative flow chart of NK cell subsets in spleen of a mouse. (B) $NK1.1^+$ cells were separated into the following subsets: DN, double negative NK cells (NK cell precursors, $CD11b^-$ $CD27^-$); R1, naïve NK cells ($CD11b^-$ $CD27^+$); R2, mature ready to kill NK cells (R2: $CD11b^+$ $CD27^+$) and R3, terminally mature predominantly cytokine producing NK cells ($CD11b^+$ $CD27^-$). (B) Ratio of R2 and R3 to R1 NK cells. Values are means ±SEM, *n*=9/group. * P<0.05

Fig. 5. Cytokine secretion by splenocytes stimulated with LPS for 24 hrs. Cytokines were measured with a cytometric bead array. Values are means ±SEM, *n*=10-11/group. * P<0.05

Fig. 6. Effect of a high folic acid diet on NK cell cytotoxicity after stimulation of splenocytes from aged mice with 15 ng/mL of recombinant mouse IL-10 for 15 hrs. Data shown for 25:1

effector to target cell ratio and is similar for the 12.5:1 effector to target cell ratio (not shown). Values are means ±SEM, *n*=10-11/group. * P<0.05

Supplementary Figures

Supplementary Figure 1. Effect of a high folic acid diet on T cell profile in splenocytes from aged mice. Percent T cells ($CD3^+$), T helper cells ($CD3^+$ $CD4^+$), and cytotoxic T cells ($CD3^+$ $CD8^+$) in the spleen. Values are means ±SEM, *n*=10-11/group. * P<0.05

Supplementary Figure 2. Effect of a high folic acid diet on cytokine secretion by splenocytes from aged mice stimulated with ConA or anti-CD3 plus anti-CD28. (A) Splenocytes were stimulated with ConA (1.5µg/mL) for 48 hrs or (B) with plate coated anti-CD3 (5µg/mL) plus soluble anti-CD28 (1µg/mL) for 48 hrs. Cytokines were measured with cytometric bead array. Values are means ±SEM, *n*=9-11/group. * P<0.05

Figure 1

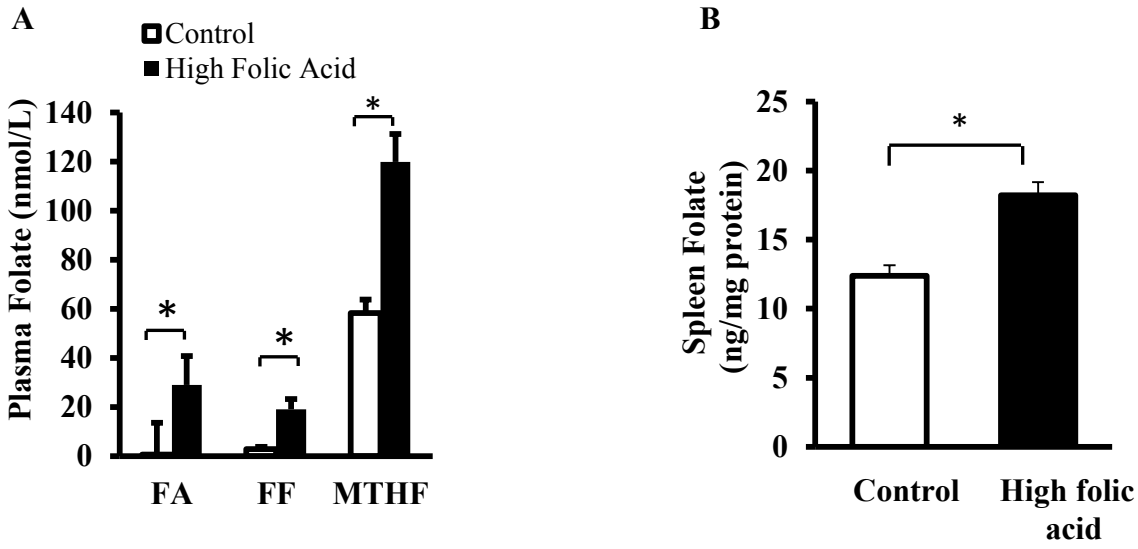

A
Control
High Folic Acid
Plasma Folate (nmol/L)
140
120
100
80
60
40
20
0
*
*
*
FA
FF
MTHF
B
Spleen Folate (ng/mg protein)
25
20
15
10
5
0
*
Control
High folic acid

Figure 2

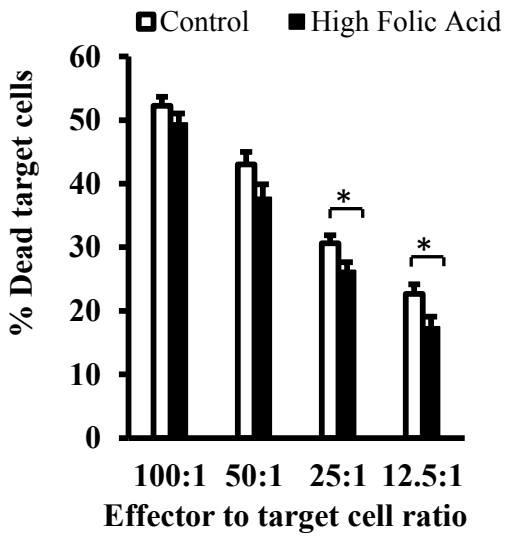

Control
High Folic Acid
% Dead target cells
60
50
40
30
20
10
0
*
*
100:1
50:1
25:1
12.5:1
Effector to target cell ratio

Figure 3

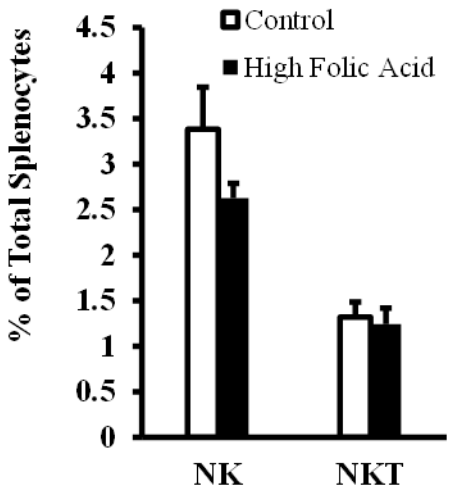

Control
High Folic Acid
% of Total Splenocytes
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
NK
NKT

Figure 4

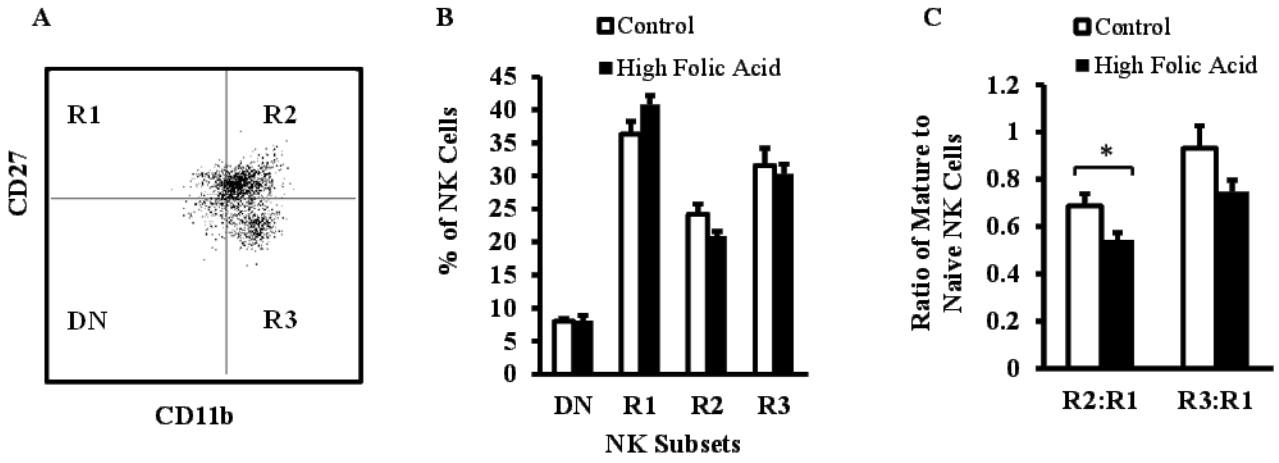

A
R1
R2
DN
R3
CD27
CD11b
B
Control
High Folic Acid
% of NK Cells
45
40
35
30
25
20
15
10
5
0
DN
R1
R2
R3
NK Subsets
C
Control
High Folic Acid
Ratio of Mature to Naive NK Cells
1.2
1
0.8
0.6
0.4
0.2
0
*
R2:R1
R3:R1

Figure 5

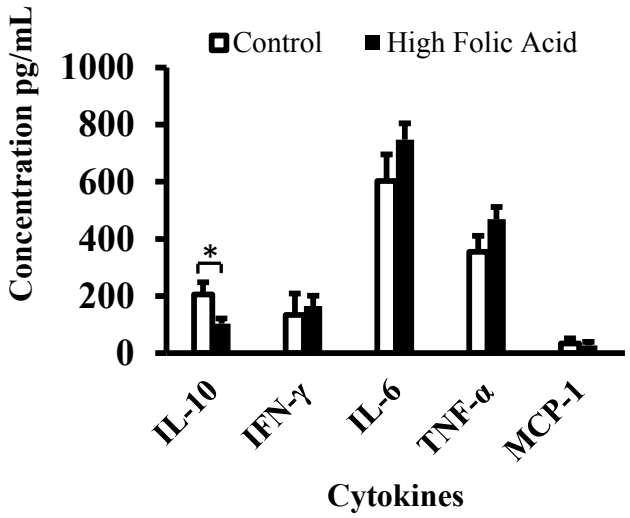
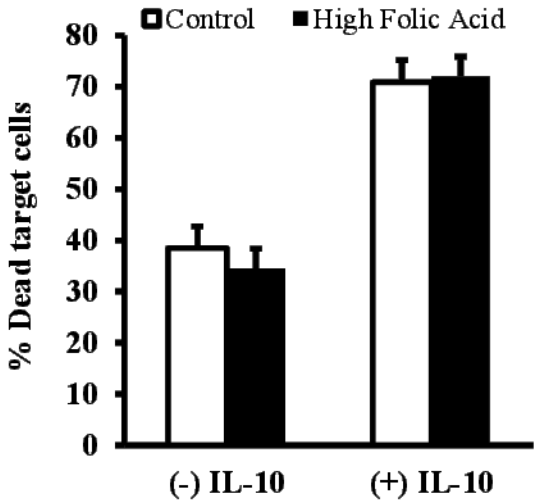
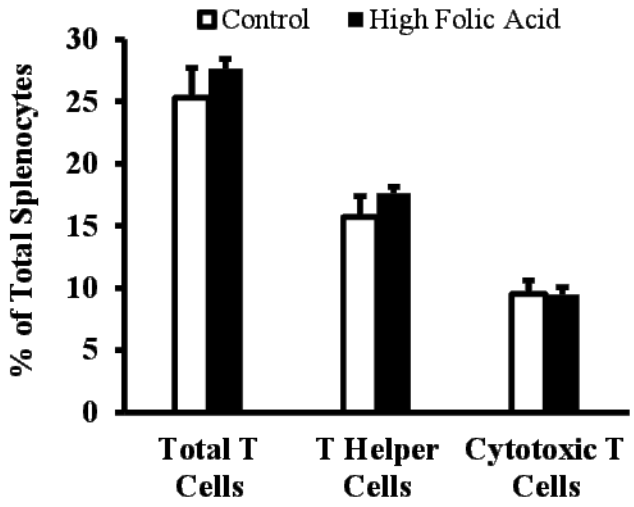

Control
High Folic Acid
Concentration pg/mL
1000
800
600
400
200
0
*
IL-10
IFN-γ
IL-6
TNF-α
MCP-1
Cytokines

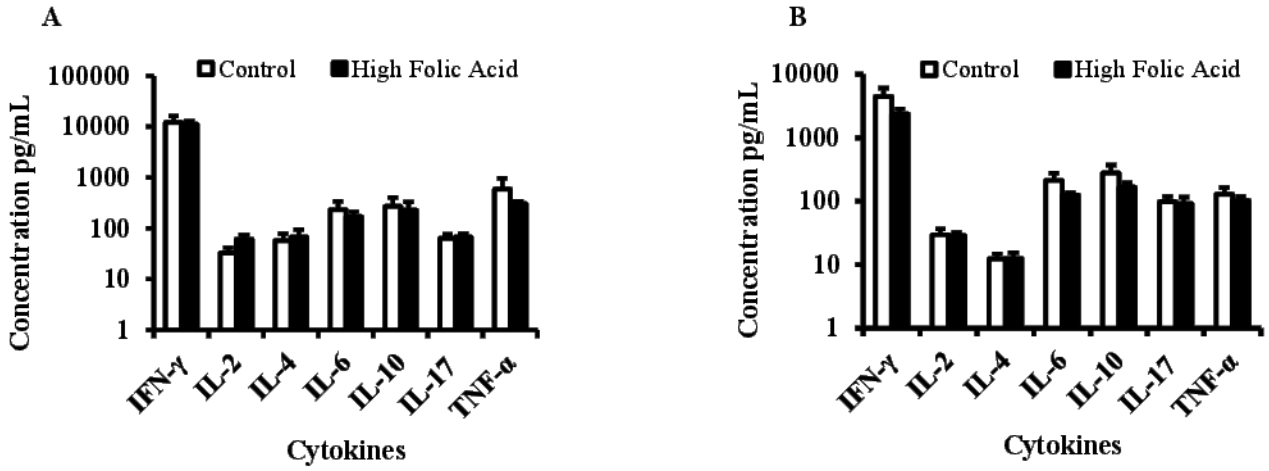
Figure 6


Control
High Folic Acid
% Dead target cells
80
70
60
50
40
30
20
10
0
(-) IL-10
(+) IL-10

Supplementary Figure 1



Supplementary Figure 2



Chapter III

Effects of a High Folic Acid Diet on Viral Titer in H1N1 Infected Aged Mice

Effects of a High Folic Acid Diet on Viral Titer in H1N1 Infected Aged Mice ☆

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Running title: High folic acid diet & influenza infection

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Keywords: Folic acid, folate, influenza infection

Abstract

We previously showed that aged mice fed a high folic acid diet had significantly lower natural killer (NK) cell cytotoxicity compared to aged mice fed a control diet. Optimal NK cell activity is important for defense against viral infections and impairment leads to increased viral infections. In this study, we determined if a high folic acid diet can result in increased severity of influenza (H1N1) infection in an aged mouse model. Female C57BL/6 mice (16-month-old) were fed an AIN-93M diet with the recommended daily allowance (1x RDA, control) or 20x RDA (high) folic acid for 3 months. Mice fed a high folic acid diet had higher viral titer in lung tissues after 2 days post-infection compared to control mice ($P=0.02$), although there was no differences after 5 days post-infection. Mice fed a high folic acid diet also had significantly lower expression of the inflammatory cytokines interleukin(IL)-6 ($P=0.012$), IL-1β ($P<0.01$), and interferon-gamma (IFN-γ) ($P=0.035$) 5 days post-infection compared to mice fed a control diet. Other cytokines including tumor necrosis factor-alpha (TNF-α), IFN-β, IL-10, IL-12 (IL-12p40 or IL-12p35), or proteins downstream to IFN-γ signaling B cell lymphoma-extra large (Bcl-xl), or myeloid cell leukemia-1 (Mcl-1) were not different between the two dietary groups. There were no differences in weight loss, NK cell cytotoxicity in spleen or lung, or lipopolysaccharide-stimulated cytokine secretion between dietary groups.

These results suggest that high folic acid intake in the aged, such as old mice in the current study, may increase viral load in an influenza infection but further research is needed to determine the mechanisms.

Keywords: Folic acid, folate, influenza infection

1. Introduction

Due to the importance of adequate folate intake in the prevention of neural tube defects, the U.S. Food and Drug Administration (FDA) mandated folic acid fortification in flour and grain products in 1998. However, in addition to consumption of multiple servings of fortified foods, approximately 39% of the U.S. population consumes multivitamin pills containing folic acid, with the majority being women over the age of 60 [1, 2] . Folic acid is the synthetic form of folate found in supplements and used in food fortification due to its stability. It requires reduction to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR), an enzyme that can be overwhelmed in case of excess intake [3], which leads to the appearance of unmetabolized folic acid in plasma [4, 5].

We previously showed that presence of unmetabolized folic acid in women older than 60 was associated with decreased natural killer cell (NK) cytotoxicity, but not in younger women [6]. This finding was replicated in an aged mouse model, revealing a causal association between excess folic acid intake and decreased NK cell cytotoxicity[7]. NK cells are innate immune cells important for fighting viral infections and cancerous cells by binding and killing infected or malignant cells. Impaired NK cell activity, as determined by the NK cytotoxicity assay, is linked to increased susceptibility to, and more severe symptoms as well as delayed recovery from diseases such as the influenza infection[8]. Studies in mice show that NK cytotoxicity decreases with age [9]. Thus, the finding that presence of unmetabolized folic acid in plasma is associated with decreased NK cytotoxicity in older women may have implications for higher risk in cancer development and susceptibility to viral infection in older populations, especially in those who take supplements.

Morbidity and mortality during influenza epidemics are particularly high among elderly people [10, 11]. Viral infection results in acute phase responses such as lethargy, fever, and weight loss [12]. Our *hypothesis* was that if NK cell function is decreased in older populations with high consumption of folic acid, it may increase susceptibility to infection.

The aim of the proposed project was to determine the effect of excess dietary folic acid on the severity of disease in an influenza infection model of mice. We hypothesized that due to decreased natural killer cell activity, aged mice fed a high folic acid diet will have greater severity of disease when infected with an influenza virus, as measured by weight loss and viral titer. From this study we expect to determine if excess folic acid consumption would potentially increase susceptibility to viral infection in older populations. Given the high prevalence of excess consumption of folic acid in the United States and many other countries that fortify foods with folic acid, the outcome of this study will have important public health implications for the aging population.

2. Materials and Methods

2.1 Influenza Infection Mouse Model.

Specific pathogen-free (7-9 months) female C57BL/6 mice were purchased from Harlan Laboratories and aged for 7 months at the Comparative Biology Unit at HNRCA on a standard chow diet (Teklad). Aged mice (16-17 months) were then housed singly in filtered cages in a controlled environment (temperature 23C, 45% relative humidity, 12-hr light-dark cycle). 22-24 mice per group were fed ad libitum with a control diet (AIN-93M diet supplemented with 2mg folic acid/kg diet) or the high folic acid diet (AIN-93M diet supplemented with 40mg folic acid/kg diet) for 3 months while their body weight was recorded weekly as diagrammed in Figure 1. After 3 months, 8 mice per diet group were administered PBS (sham control group) and 14-18 mice per diet group were infected with the influenza A/Puerto Rico/8/34 (H1N1) virus according to the method of Yetter et al [13], with modifications as previously reported [14-16]. Briefly, mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg)/xylazine (1.25mg/kg) solution before they were infected intranasally with 0.02 hemagglutinin units of influenza H1N1 diluted in 0.05 mL PBS [16]. Mice continued to consume their experimental diets and were weighed daily before being euthanized on day 0, 2, and 5 (7-9 mice per diet group per time point) post-infection.

2.2 Spleen and lung cell isolation.

Mice were euthanized by CO_2 asphyxiation followed by exsanguination through cardiac puncture, and blood was used for plasma isolation. Spleen was removed aseptically and single-cell suspensions were prepared as previously described [17]. Spleens were placed in sterile RPMI 1640 medium (Lonza, MA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 25 mmol/L HEPES, 2 mmol/L glutamine, 100 kU/L penicillin, and 100 mg/L streptomycin (Life Technologies, CA). To make single cell suspension, spleen was disrupted between two sterile frosted glass slides. The cells were collected by centrifugation at 300G for 10 min and resuspended in red blood cell lysis buffer (Sigma, MO) to remove red blood cells. After washing with PBS and removing cell debris by filtering through 40 μM nylon cell strainer (BD Pharmingen) splenocytes were counted using an Accuri C6 flow cytometer (BD Accuri Cytometers, MI) and resuspended at appropriate densities in culture medium for the assays.

Lungs were removed aseptically and lung lymphocytes were isolated as previously described [16]. In this procedure, lungs were cut into small pieces and incubated in Iscove's medium (Life Technologies, CA) with 10% FBS and 40U/mL of collagenase (Sigma, St Louis, MO) for 1 hour at 37°C. Lung tissue were ground and strained through a 60-mesh stainless steel sieve and centrifuged to collect the supernatant containing the virus. The lung supernatant was stored at -80C for viral titer measurements. Lung lymphocytes were counted using an Accuri C6 flow cytometer (BD Accuri Cytometers, MI) and resuspended in appropriate media for the assays.

2.3 Viral Titer.

Single cell suspensions were prepared from lung parenchyma and spleen. Lung virus titer were measured by inoculating lung supernatant into Madin Darby canine kidney cells and calculating $TCID_{50}$ (50% tissue culture infectious dose) by using the method of Reed and Muench [18].

2.4. Preparation of target cells.

YAC-1 cells, a murine T- lymphoma cell line sensitive to NK cell killing, were grown in complete RPMI 1640 medium prepared as above and maintained at 37°C in 5% CO_2. Starting 5 days prior to the assay, cells were sub-cultured every 24 h to ensure that they were in the log phase. YAC-1 cells were stained with 10 nM of carboxyfluorescein succinimidyl ester (CFSE, eBioscience, CA) for 10 minutes at room temperature in the dark, washed twice in PBS with 2% FBS and resuspended to a final density of 10^6 cells/mL in RPMI 1640 medium.

2.5. NK cytotoxicity assay.

The flow cytometric assay described by McGinnes et al [19] was used with modifications as described by Cao et al. [20]. All reagents were purchased from eBioscience. Splenocytes and lung lymphocytes (10^7/tube) were stained with allophycocyanin (APC)-conjugated anti-CD3 antibody and phycoerythrin (PE)-conjugated anti-NK1.1 antibody to identify NK cells ($CD3^-$ $NK1.1^+$). Stained splenocytes were then incubated with CFSE-labelled YAC-1 cells (target cells) at effector-to-target (E:T) cell ratios of 50:1, 25:1, and 12.5:1 for 3 h at 37C with 5% CO_2. After the incubation was complete, 7-amino-actinomycin D (7-AAD) was added to each tube to a final concentration of 1.11 μg/mL. Dead YAC-1 cells, which were identified as 7-AAD and CFSE double positive cells, were counted using an Accuri C6 flow cytometer and acquired data were analyzed with FlowJo 7.6 software (Treestar Inc., OR). CFSE stained YAC-1 cells incubated in the absence of splenocytes were used to determine spontaneously dead target cells. Percent specific target cell death (cytotoxicity) was then expressed as: 100 x {[dead YAC-1 Cells (%) – spontaneously dead YAC-1 Cells (%)]/ [100 – spontaneously dead YAC-1 target cells (%)]}.

2.6. Immunophenotyping.

Splenocyte and lung lymphocyte populations were determined using flow cytometry. Splenocytes and lung lymphocyte were blocked using anti-CD16/32 antibodies and then stained using the following fluorochrome-conjugated antibodies: PE or APC-anti-CD3, APC-anti-CD4, fluorescein isothiocyanate (FITC) anti-CD8 and PE-anti-NK1.1 (eBioscience), to identify all T cells ($CD3^+$), T-helper cells ($CD3^+$ $CD4^+$), cytotoxic T cells ($CD3^+$ $CD8^+$), NK cells ($CD3^-$ $NK1.1^+$) and natural killer T (NKT) cells which have the properties of both T cells and NK cells ($CD3^+$ $NK1.1^+$). The stained cells were then analyzed using Accuri C6 flow cytometer and acquired data analyzed with FlowJo 7.6 to determine the immune cell phenotype.

NK cell subsets were identified as previously described [9] using the following fluorochrome conjugated antibodies: APC-anti-CD3, PE-anti-NK1.1, FITC-anti-CD27 and PerCP-Cy5.5-anti-CD11b. All staining reactions included isotype controls to detect non-specific background signals. The antibodies and the respective isotype controls were purchased from eBioscience. The stained cells were analyzed using an Accuri C6 flow cytometer and the data were analyzed with FlowJo 7.6.

2.7. Lung Cytokine production.

Production of cytokines involved in inflammation including IFN –β, IFN-γ, IL-1β, IL-10, IL-6, IL-2, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) in the supernatants from isolated lung lymphocytes were determined using commercial Elisa kits (eBioscience).

2.8. LPS Stimulated Cytokine production.

Splenocytes in complete RPMI 1640 medium were stimulated in separate reactions with bacterial lipopolysaccharide (LPS, 1μg/mL) for 24 h. At the end of the incubation, cell-free supernatants were collected and stored at -80°C until analysis. The concentration of IL-6, IL-10, TNF-α, MCP-1, and IFN-γ in the supernatants from LPS stimulated cells were determined using commercial Elisa kits (eBioscience).

2.9. Gene expression cytokines in lung tissue.

A piece of lung was frozen in TRIzol reagent until it was ready for RNA isolation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA was reverse transcribed with SuperScript IV First-Strand Synthesis System kits (Life Technologies) and mRNA levels for the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the target genes were assayed by real-time PCR in an Applied Biosystems 7300 Real Time PCR system using Power SYBR Green PCR Master Mix and the following primer sets: *GAPDH* AGGTCGGTGTGAACGGATTTG (forward), TGTAGACCATGTAGTTGAGGTCA (reverse) [21]*; IL-10* GCTCTTACTGACTGGCATGAG (forward), CGCAGCTCTAGGAGCATGTG (reverse) [22]*; TNF-α* GGTCTGGGCCATAGAACTGA (forward), CAGCCTCTTCTCATTCCTGC (reverse) [23]*; IFN-γ* ATGAACGCTACACACTGCATC (forward), CCATCCTTTTGCCAGTTCCTC (reverse) [24]*; IFN-β* CAGCTCCAAGAAAGGACGAAC (forward), GGCAGTGTAACTCTTCTGCAT (reverse) (PrimerBank ID 6754304a1) [25]*; IL-1β* CCCTGCAGCTGGAGAGTGTGGA (forward), TGTGCTCTGCTTGTGAGGTGCTG (reverse) [26]*; IL-6* TAGTCCTTCCTACCCCAATTTCC (forward), TTGGTCCTTAGCCACTCCTTC (reverse) [27]*; IL-12p35* TACTAGAGAGACTTCTTCCACAACAAGAG (forward), TCTGGTACATCTTCAAGTCCTCATAGA (reverse) [28]*; IL-12p40* GACCATCACTGTCAAAGAGTTTCTAGAT (forward), AGGAAAGTCTTGTTTTTGAAATTTTTTAA (reverse) [28]*; Bcl-xl* AACATCCCAGCTTCACATAACCCC (forward), GCGACCCCAGTTTACTCCATCC (reverse) [29]*; Mcl-1* TGTAAGGACGAAACGGGACT (forward), AAAGCCAGCAGCACATTTCT (reverse) [30]. The delta delta CT method was used to determine expression of target genes relative to sham control mice, using GAPDH as the internal control gene.

2.10. Folate analysis.

Total folate from spleen was determined using a microbial assay with *Lactobacillus casei* [31]. Protein concentration of the spleen extract used for folate assay was determined by Bradford method [32] using Bio-Rad protein assay reagent (Bio-Rad, CA).

2.11. Statistical Analysis.

Repeated-measures ANOVA were used for body weight because data were collected daily for the same animals until day 5 post-infection. Student's t-test was used to determine the effect of diet on viral titer and other secondary variables within infection days. Statistical significance were defined as $P<0.05$. All statistical calculations were performed using SAS v9.2 (SAS Institute; Cary, NC).

3. Results

3.1. Animal Study

Of the 64 female C57BL/6 started for the study, 48 mice survived after being aged for 7 months. These mice were randomized to control (1x RDA folic acid) or high folate (20x RDA of folic acid) diet for 3 months. Mice were then infected with influenza A/Puerto Rico/8/34 (H1N1) virus or PBS (sham mice) and their body weight was recorded until day 2 and day 5 post-infection, at which times mice were euthanized and samples were collected for analysis.

3.2. Folate Concentration in Spleen and Lung

Mice fed a high folic acid diet had significantly higher concentration of folate in spleen in non-infected mice ($P=0.0048$) but there were no differences in folate concentration in spleen after day 2 or day 5 post-infection (Figure 2A). Mice fed a high folic acid diet also had higher folate concentration in lung compared to control mice in non-infected mice ($P=0.0159$) (Figure 2B). There was a trend towards higher concentrations of folate in lungs of high folic acid mice at day 2 post-infection compared to mice fed a control diet ($P=0.0613$) (Figure 2B). There was no difference in lung folate concentration at day 5 post-infection.

3.3. Viral Titer

Mice fed a high folic acid diet had significantly higher viral load in lung compared to mice on control diet after 2 days post-infection after adjusting per million of pulmonary cells (P=0.02) (Figure 2). However, there were no significant differences between diets in viral load in lung after 5 days post-infection.

3.4. Weight Loss, NK Cytotoxicity, NK Maturation

There were no significant differences in weight loss (Figure 4), and NK cytotoxicity in spleen or lung, between control and high folic acid dietary groups at day 2 and 5 post-infection (Figure 5A and B). There were no differences in percent total NK, NKT, or CD3 (T cell surface marker) lymphocytes between dietary groups in sham (day 0, uninfected), day 2, or day 5 post-infection (Figure 6).

In our preliminary pilot study, we found that mice fed a high folic acid diet had reduced mature to naïve NK cell ratio which may contribute to their decreased NK cytotoxicity [7]. In this study, we found that there were no differences in NK maturity between diets in sham mice. This may potentially be due to our reduced sample size (n=8 per group) compared to previous studies (n=10-12 mice per group). However, 2 days post-infection, mice fed a high folic acid diet had more mature cells (R2 subset) in spleen compared to control mice although the difference was not significant (P=0.0589) (Figure 7). At day 5 post-infection, there was no difference in mature NK cell subsets but mice fed a high folic acid diet had a significantly higher percent of naïve NK cell in spleen compared to mice fed a control diet (P=0.0087) suggesting an alteration in NK maturity after influenza infection even in the absence of difference in NK cell function, i.e., NK cell cytotoxicity.

3.5. Lung Cytokine Secretion, LPS Stimulated Cytokine Secretions

Lung tissue supernatant was saved after lung lymphocytes were isolated from lung tissue. However, due to the dilution of lung tissue supernatant during the lung lymphocyte isolation process, we were unable to detect lung cytokine secretions such as IFN-γ, IL-6, TNF-α, IL-1β, and IL-10 using the available ELISA kits, even after the samples were concentrated by five-fold. The only detectable cytokine was IL-2 but its concentrations were not different between the two diet groups (Figure 8).

After lung and spleen lymphocytes were stimulated ex vivo for 24 hours with LPS, supernatant was collected and cytokine concentrations were measured with ELISA kits. There were no differences in IFN-γ, IL-6, TNF-α, IL-1β, and IL-10 concentrations between the diets groups (Figure 9A and B).

3.6. Gene expression of Inflammatory Cytokines in Lung

Since lung supernatant was too dilute to detect inflammatory cytokines, cytokine mRNA expression was measured in lung tissue. There were no differences in expression of IL-6, IFN-γ, and IL-1β between the two diet groups in non-infected and day 2 post-infected mice. However, expression of IL-6, IFN-γ, and IL-1β were significantly lower in high folic acid mice compared to control mice after 5 days of infection ($P<0.05$) (Figure 10A-C). We also found that there was a significant diet by time interaction for IL-6 ($P=0.0231$); while IL-6 expression was increased in control mice after infection, no change was observed in the high folic acid mice (Figure 10A). There were no other significant diet by infection day interactions for expression of the other cytokines in lung lymphocytes. Expression of TNF-α and IL-10 was not different between the two diet groups in non-infected, day 2 or day 5 post-infection (Figure 10D, E).

Since we found lower secretions of IL-6 in the high folic acid group compared to the control group after 5 days of infection, we wanted to determine if a high folic acid diet also decreased expression of proteins downstream of IL-6. Previous studies have shown that during an influenza infection, IL-6 secretion stimulates transcription of anti-apoptotic molecules Bcl-x and Mcl-1 in neutrophils [33]. This mechanism allows neutrophils to survive and combat the influenza infection in lungs. We measured Bcl-xl and Mcl-1 gene expression in lung cells and saw no statistically significant differences between the two diet groups within infection days for either gene (Figure 10 G, H).

We wanted to investigate if other interferons were affected by the high folic acid diet and found that after 5 days of infection, mice fed a high folic acid diet had strong trend toward a lower expression of IFN-β ($P=0.0678$) (Figure 10F), which is important for immune mobilization to fight the influenza virus. Since a high folic diet led to decreased IFN-γ and a trend towards decreased IFN- β expression, we sought to determine if a cytokine that regulates interferons was also affected in the high folic acid mice. IL-12 is

a cytokine that increases after influenza infection which then stimulates interferon expression and secretion. It is coded by two genes, IL-12p35 and IL-12p40, but we found no diet effect on expression of either gene expressions (Figure 10 I, J).

4. Discussion

We showed that aged mice fed a high folic acid diet had higher folate concentrations in spleen and lung before infection, but this difference disappeared after infection suggesting that the influenza infection may affect localization of total folate in tissue (Figure 2). Mice fed a high folic acid diet had significantly higher viral titer in lung tissues 2 days after infection compared to control mice, although no differences was found 5 days post-infection (Figure 3). While all mice lost weight after infection, there was no significant difference in weight loss between the two diet groups (Figure 4). Mice fed a high folic acid diet also had significantly lower expression of inflammatory cytokines 5 days post-infection compared to mice fed a control diet (Figure 10). There was a shift in maturation of NK cells in splenocytes with less mature natural killer (NK) cells seen in mice fed a high folic acid after 5 days of infection (Figure 7). There was no difference in NK cell cytotoxicity in splenocytes or lung lymphocytes (Figure 5). It is possible that the study was not powered to detect a difference in NK cell cytotoxicity since there was an unexpectedly high mortality rate due to aging prior to the start of the experiment.

The combined evidence suggests that a high folic acid diet may alter viral titer in lungs of aged mice in the early stages of infection (2 days post-infection) but this is not reflected in the primary clinical outcome of weight loss or functional assays such as NK cell cytotoxicity. Expression of inflammatory cytokines increased after infection in control mice, but mice fed a high folic acid diet did not have similar increases. It is important for inflammatory cytokines to increase after infection in order to activate immune cells for viral load clearance and recovery from infection. The failure to increase inflammatory cytokines in response to infection in the high folic acid mice may lead to hindered viral load clearance and longer duration of flu. On the other hand, over production of inflammatory cytokines may also lead to increased lung pathology and tissue damage. Since lung pathology was not measured in this study, and outcomes were not measured at day 7 and 9 post-infection to determine if there was a difference in viral

clearance between the two dietary interventions, further studies are needed to elucidate whether a high folic acid diet is beneficial or harmful in terms of fighting influenza infection.

This study shows that excess folic acid consumption may potentially increase susceptibility to viral infection in older populations. Since 90% of deaths due to the flu occur in those older than 65 years old, it is important to screen for other risk factors that may increase this population's vulnerability to the flu [34]. In addition, folic acid intakes are highest in those aged 50 years or older, with 5% of this group exceeding the Tolerable Upper Intake Level of 1mg/day for folate [35]. With the high prevalence of excess consumption of folic acid in the United States and many other countries that fortify with folic acid, this study will have important public health implications for the aging population.

Conflict of interest

The authors declare they have no conflict of interest

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Figure Legends

Fig. 1. Influenza Infection Model of Mice. Mice were aged until they are 16-17 months old, and then fed ad libitum with a control diet (2mg folic acid/kg diet) or high folic acid diet (40mg folic acid/kg diet) for 3 months and weighed weekly. After 3 months, 8 mice per diet group were treated with PBS (sham control group) and 14-18 mice per diet group were infected with the influenza A/Puerto Rico/8/34 (H1N1) virus as described in the methods section.

Fig. 2. Folate Concentration in Spleen and Lung. Folate concentration in spleen (A) and lung (B) measured by microbial assay. Values are means ± standard deviation with N=5-10 per group.

Fig. 3. Effect of high folic acid diet on lung viral titer. At 2 and 5 days post-infection, lungs were homogenized and pulmonary cells extracted. Viral titer of lung supernatant was measured using the Madin-Darby canine kidney assay and expressed as the 50% tissue culture infectious dose (TCID50) per million pulmonary cells. Values are means ± standard deviation with N=3-7 per group.

Fig. 4. Change in weight with influenza infection. Percent weight loss during infection for sham mice and mice infected for 5 days in control and HF diet. There were no differences in weight loss between diet groups in the day 5 post infection group for any of the days. Values are means ± standard deviation with N=3-7 per group.

Fig. 5. NK Cytotoxicity in mice fed a control (1x RDA) or high folic acid (20x RDA) diet. Splenocytes (A) or Lung Lymphocytes (B) were isolated and incubated with YAC-1 target cells at 100:1, 50:1, 25:1, and 12.5:1 ratio of effector to target cells. Dead target cells (YAC-1 cells, CFSE+, 7-AAD+) were quantified by flow cytometry. Values are means ± standard deviation, *n*=4-8/group.

Fig. 6. Percent NK Cells in splenocytes. Percent of NK ($CD3^-$ $NK1.1^+$), NKT ($CD3^+$ $NK1.1^+$), or T cells ($CD3^+NK1.1^-$) cells in total splenocytes quantified by flow cytometry. Values are means ± standard deviation, *n*=4-8/group.

Fig. 7. NK cell naïve and mature subsets in spleen of mice fed a control (1xRDA) or 20xRDA folic acid diet for three months. In mice, mature and naïve NK cells are distinguished based on the presence of the surface markers CD11b and CD27 [23]. Double negative (DN, CD11b- CD27-) cells are NK cell precursors without effector function, CD11b- CD27+ (R1 subset) are naïve and have reduced effector functions, CD11b+ CD27+ (R2 subset) are mature NK cells capable of killing target cells and secreting cytokines, and CD11b+ CD27- (R3 subset) are terminally differentiated, mature NK cells that predominantly secretes cytokines [11, 23-25]. The sequence of maturation of NK cells in mice is as follows: DN>R1>R2>R3 [25]. Values are means ± standard deviation, n=4-8/diet group

Fig. 8. Concentration of interleukin-2 (IL-2) in lung supernatant. The cytokine was measured with an ELISA kit. Values are means ± standard deviation, n=5-10/group.

Fig. 9. Concentration of cytokines secreted when splenocytes (A) or lung lymphocytes (B) were stimulated with LPS for 24 hrs. Cytokines were measured with individual ELISA kits. Values are means ± standard deviation, *n*=5-10/group. * $P<0.05$

Fig. 10. Relative mRNA expression of cytokines in lung tissue**.** Expression of A) Interleukin-6 (IL-6) B) Interferon-gamma (IFN-γ) C) Interleukin-1β (IL-1β) and D) Tumor Necrosis Factor-Alpha (TNF-α), E) Interleukin-10 (IL-10), F) Interferon-Beta (IFN-β), G) B-cell lymphoma-extra large (Bcl-xl), H) Myeloid Cell Leukemia-1 (Mcl-1), I) Interleukin-12 p40 subunit (IL-12p40) and J) Interleukin-12 p35 subunit (IL-12p35) were measured and determined relative to GAPDH. mRNA expressions were then normalized to the control, non-infected mice group. Values are means ± standard deviation with N=3-7 per group.

Figure 1

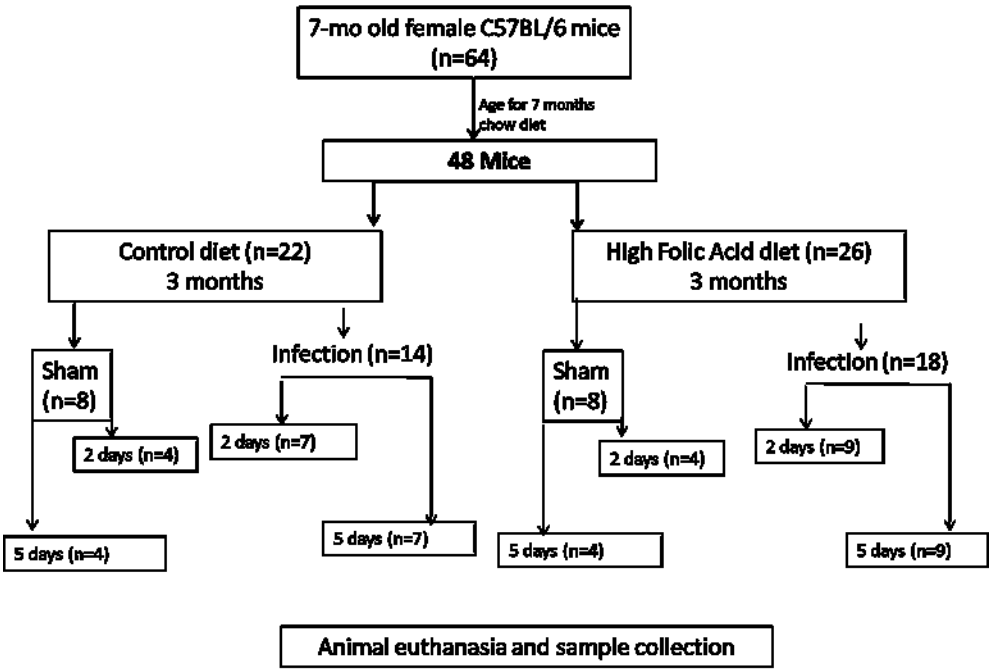

7-mo old female C57BL/6 mice (n=64)
Age for 7 months chow diet
48 Mice
Control diet (n=22) 3 months
High Folic Acid diet (n=26) 3 months
Sham (n=8)
Infection (n=14)
Sham (n=8)
Infection (n=18)
2 days (n=4)
2 days (n=7)
2 days (n=4)
2 days (n=9)
5 days (n=4)
5 days (n=7)
5 days (n=4)
5 days (n=9)
Animal euthanasia and sample collection

Figure 2

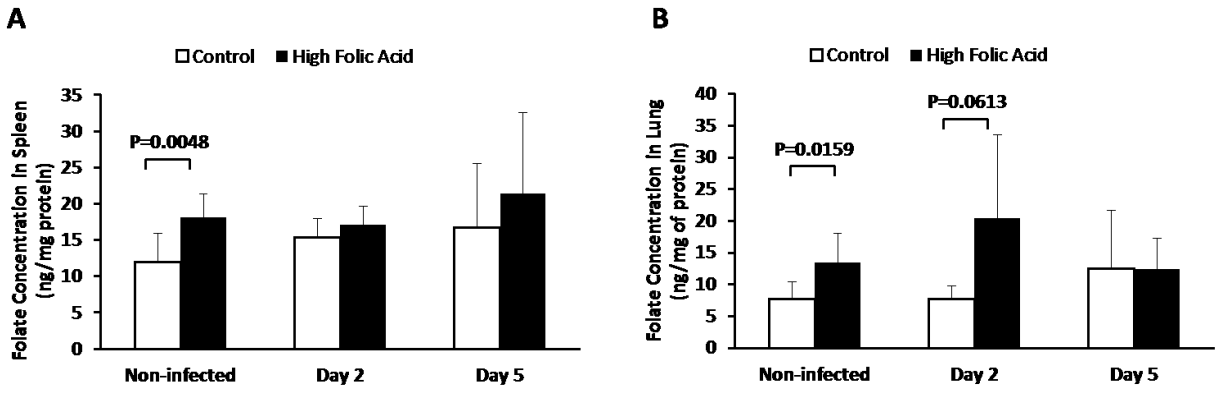

A
Control
High Folic Acid
Folate Concentration In Spleen (ng/mg protein)
35
30
25
20
15
10
5
0
P=0.0048
Non-infected
Day 2
Day 5
B
Control
High Folic Acid
Folate Concentration In Lung (ng/mg of protein)
40
35
30
25
20
15
10
5
0
P=0.0159
P=0.0613
Non-infected
Day 2
Day 5

Figure 3

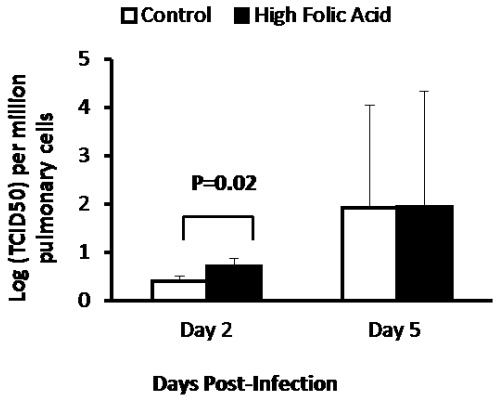


Figure 4

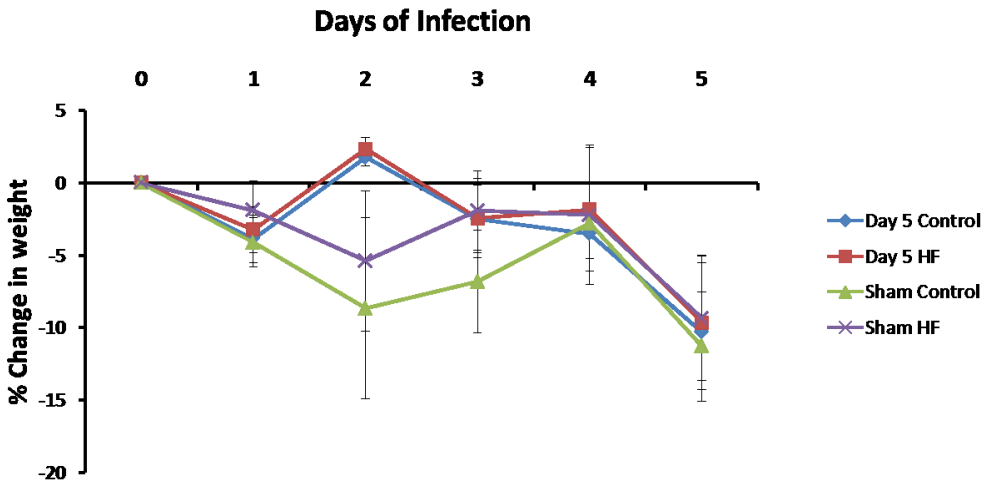

Days of Infection
0
1
2
3
4
5
% Change in weight
5
0
-5
-10
-15
-20
Day 5 Control
Day 5 HF
Sham Control
Sham HF

Figure 5

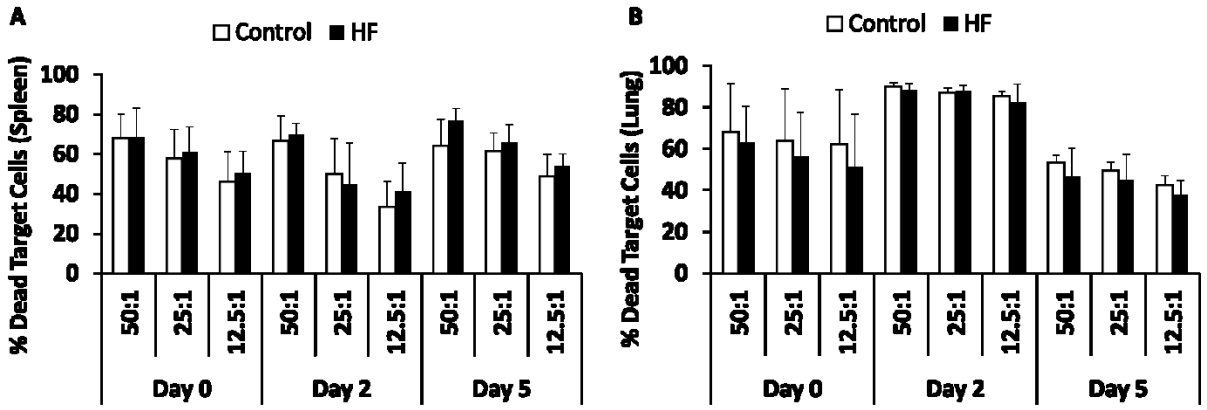

A
Control
HF
% Dead Target Cells (Spleen)
100
80
60
40
20
0
50:1
25:1
12.5:1
Day 0
Day 2
Day 5
B
Control
HF
% Dead Target Cells (Lung)
Day 0
Day 2
Day 5

Figure 6

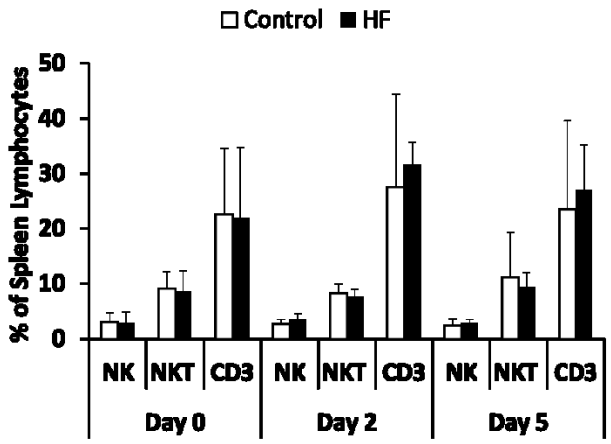

Control
HF
% of Spleen Lymphocytes
50
40
30
20
10
0
NK
NKT
CD3
NK
NKT
CD3
NK
NKT
CD3
Day 0
Day 2
Day 5

Figure 7

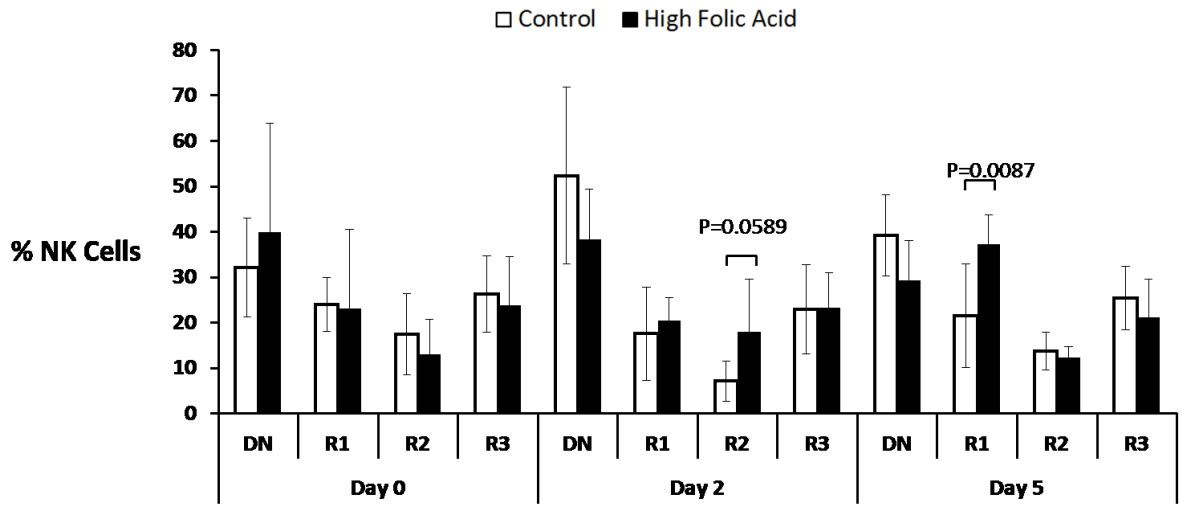


Figure 8

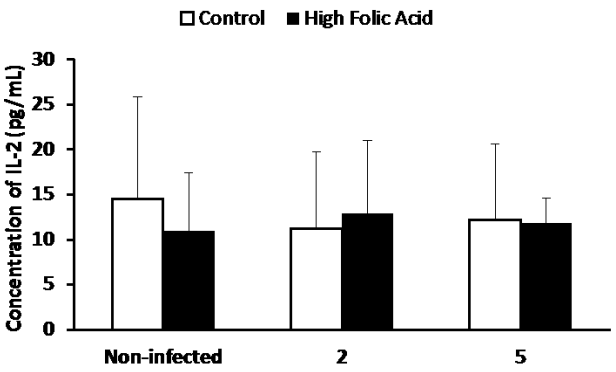
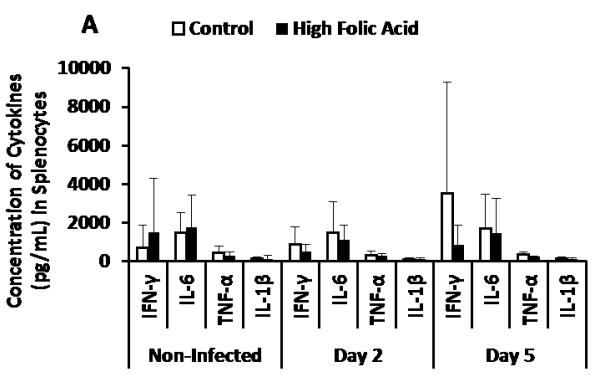

Control
High Folic Acid
Concentration of IL-2 (pg/mL)
30
25
20
15
10
5
0
Non-infected
2
5

Figure 9


A
Control
High Folic Acid
Concentration of Cytokines (pg/mL) In Splenocytes
10000
8000
6000
4000
2000
0
IFN-γ
IL-6
TNF-α
IL-1β
Non-Infected
Day 2
Day 5

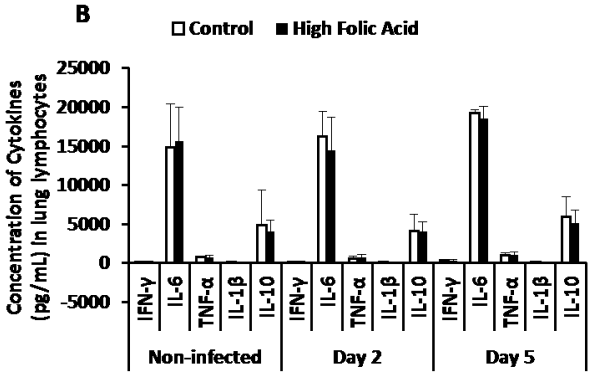
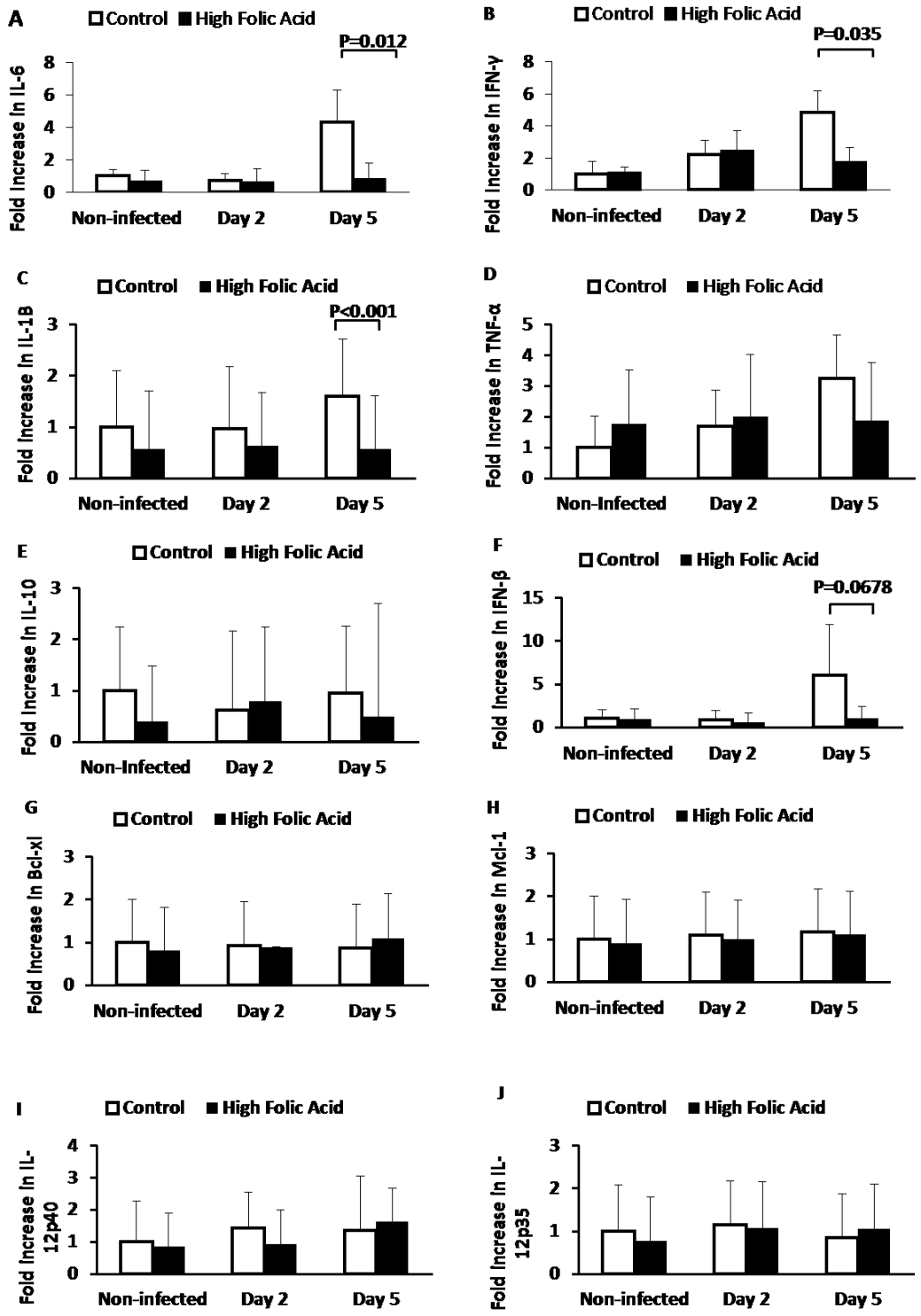

B
Control
High Folic Acid
Concentration of Cytokines (pg/mL) In lung lymphocytes
25000
20000
15000
10000
5000
0
-5000
IFN-γ
IL-6
TNF-α
IL-1β
IL-10
Non-infected
Day 2
Day 5

Figure 10


A
Control
High Folic Acid
P=0.012
Fold Increase In IL-6
Non-infected
Day 2
Day 5
B
Control
High Folic Acid
P=0.035
Fold Increase In IFN-γ
Non-infected
Day 2
Day 5
C
Control
High Folic Acid
P<0.001
Fold Increase In IL-1B
Non-infected
Day 2
Day 5
D
Control
High Folic Acid
Fold Increase In TNF-α
Non-Infected
Day 2
Day 5
E
Control
High Folic Acid
Fold Increase In IL-10
Non-Infected
Day 2
Day 5
F
Control
High Folic Acid
P=0.0678
Fold Increase In IFN-β
Non-infected
Day 2
Day 5
G
Control
High Folic Acid
Fold Increase In Bcl-xl
Non-infected
Day 2
Day 5
H
Control
High Folic Acid
Fold Increase In Mcl-1
Non-infected
Day 2
Day 5
I
Control
High Folic Acid
Fold Increase In IL-12p40
Non-infected
Day 2
Day 5
J
Control
High Folic Acid
Fold Increase In IL-12p35
Non-infected
Day 2
Day 5

Chapter IV

Transcobalamin II 776C>G Polymorphism and Peripheral Neuropathy in Elders

Transcobalamin II 776C>G Polymorphism and Peripheral Neuropathy in Elders

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Conflict of interests/disclosures: None

Word count: 2620

Abstract

BACKGROUND: The 776C>G polymorphism of the vitamin B12 transport protein transcobalamin II gene (TCN2, rs1801198, Pro259Arg) is associated with lower holotranscobalamin concentration in plasma. This can reduce the availability of vitamin B12 to tissues, even when vitamin B12 intake is adequate. Clinical outcomes associated with vitamin B12 insufficiency are worsened by high folate intake.

OBJECTIVE: To determine the association of *TCN2* 776C>G polymorphism and peripheral neuropathy with emphasis on folate intake in elders with normal plasma concentration of vitamin B_{12}.

DESIGN: The study participants (n=171) were from a cohort of community-based, home-bound elderly participants aged ≥60 y who underwent evaluation by physicians including assessment for peripheral neuropathy. The participants were administered a food frequency and general health status questionnaires, anthropometric measurements were taken and a fasting blood sample was collected.

METHODS: *TCN2* 776C>G polymorphism was determined by allelic discrimination assay. Peripheral neuropathy was diagnosed after neurological examination and folate intake assessed using a food frequency questionnaire.

RESULTS: Prevalence of homozygosity for the variant allele of the *TCN2* 776C>G polymorphism was 17.2% in this cohort. Odds for neuropathy were 3 fold higher for GG genotypes when compared to CC genotypes (odds ratio: 3.33; 95% CI: 1.15, 9.64). When folate intake was above twice the Recommended Dietary Allowance (800 μg), GG genotypes had 6.9 fold higher odds for neuropathy compared to CC genotype (odds ratio: 6.9; 95% CI: 1.31, 36.36). But there was no difference between the genotypes in the odds for peripheral neuropathy when folate intake was ≤800 μg (odds ratio: 1.5; 95% CI: 0.18, 12.33).

CONCLUSIONS AND RELEVANCE: *TCN2* 776C>G polymorphism was associated with increased odds for peripheral neuropathy in elderly despite normal vitamin B_{12} status, especially if their folate intake was in excess of twice the Recommended Dietary Allowance. Since the mean folate intake in the US is more than twice the Recommended Dietary Allowance among those ≥ 51 years of age, these results are relevant to a large population of older adults who are at increased risk for neurological impairment due to the *TCN2* polymorphism.

Introduction

Transcobalamin II is a vitamin B_{12} binding protein that transports the vitamin into peripheral tissues.[1] The 776C>G single nucleotide polymorphism in the gene for transcobalamin, *TCN2*, (rs1801198) results in a Pro259Arg substitution in the transcobalamin II protein.[2] This polymorphism affects binding of vitamin B_{12} by transcoblamin II and is associated with lower concentration of holotranscobalamin (vitamin B_{12}-bound transcobalamin) in plasma and can thus affect the availability of vitamin B_{12} to the tissues.[3,4] There are 2 vitamin B_{12} dependent reactions in mammalian cells; synthesis of methionine from homocysteine and methyltetrahydrofolate catalyzed by the enzyme methionine synthase, and conversion of methyl malonyl CoA to succinyl CoA by methyl malonyl CoA mutase. Methionine is the precursor of S-adenosyl methionine, the biological methyl donor for methylation reactions in the cell. S-adenosyl homocysteine generated during every methylation reaction is hydrolyzed to homocysteine. When vitamin B_{12} availability is limited, homocysteine and methyl malonic acid concentrations increase in plasma. Thus, total homocysteine and methylmalonic acid in plasma serve as functional biomarkers for vitamin B_{12} status in tissues. The GG genotype of the 776C>G polymorphism of *TCN2* is associated with higher concentration of homocysteine in individuals with lower plasma vitamin B_{12}.[5] In a population with normal plasma values for vitamin B_{12}, the concentration of methyl malonic acid was higher in GG genotypes.[4] These studies indicate that the *TCN2* polymorphism is functional and affects availability of vitamin B_{12} in tissues for metabolic reactions.

Lack of sufficient vitamin B_{12} is associated with peripheral neuropathy, anemia, depression and cognitive impairment.[6-8] Peripheral neuropathy with characteristic features is a frequent clinical outcome in vitamin B_{12} deficiency, which has been observed even in the absence of anemia or macrocytosis.[9] It has also been reported that the elderly may present with clinical conditions and elevated concentrations of plasma biomarkers associated with vitamin B_{12} insufficiency when their plasma concentration of vitamin B_{12} is low but in the normal range.[6,7,10,11] In this study we

investigated the relationship between the *TCN2* polymorphism and peripheral neuropathy in participants aged ≥ 60 y with normal plasma concentration of vitamin B_{12}. Early reports on folic acid supplementation of vitamin B_{12} deficient patients have shown that folic acid ameliorates the megaloblastic anemia and hematological abnormalities seen in severe vitamin B_{12} deficiency, but has no effect on neurological conditions associated with the deficiency.[12] Current evidence suggests that excess folate intake worsens the clinical and metabolic manifestations of vitamin B_{12} insufficiency as indicated by the increase in the prevalence of anemia and cognitive dysfunction and the concentration of homocysteine and methyl malonic acid in plasma.[13-17] Hence we also determined the effect of folate intake on the association between the *TCN2* polymorphism and peripheral neuropathy.

Methods

Study Population

The study samples consisted of participants from the Nutrition, Aging, and Memory in Elders (NAME) study, which is a cohort of community-based, home-bound elderly participants aged ≥60 y recruited from the Boston area as described in detail previously.[18] Participants were recruited from Boston's three Aging Services Access Points, which are home care agencies that provide services to the elderly to facilitate independent living. Informed consent for all tests including genotyping was obtained. A subset of 366 participants from the total of 1246 participants underwent evaluation by physicians including assessment for peripheral neuropathy, and gait and balance at Tufts Medical Center. The participants were administered food frequency and general health status questionnaires, anthropometric measurements were taken and a fasting blood sample was collected. Participants were excluded from the current study if data were missing for *TCN2* genotype (n=53) or any of the covariates (n=86), they had a stroke (n=43) or had plasma vitamin B_{12} concentration below 200 pg/mL (n=2) indicative of deficiency, or above 950 pg/mL (n=11), which may be indicative of malignancies or abnormalities.[19-21] 171 participants who underwent evaluation by the physicians met the criteria for the current study. This study was approved by the Institutional Review Board at Tufts University and Tufts Medical Center.

Blood Analyses

Blood samples were collected after at least 7 hours of fasting. Concentrations of vitamin B_{12} and folate in plasma were measured using Immulite 1000 assay (Siemens Healthcare Diagnostics, NJ), total plasma homocysteine was determined by a high performance liquid chromatography,[22] and serum creatinine by a modified Jaffe reaction.[23] Plasma concentration of pyridoxal 5'-phosphate, the functional form of vitamin B_6 was determined by a tyrosine decarboxylase

apoenzyme method.[24] Blood glucose was measured using a hexokinase method using a Beckman Coulter AU400e instrument (Beckman Coulter, Inc., CA).

DNA Isolation and Genotyping

DNA was isolated using QIAmp DNA Blood Mini kit (Qiagen, CA). *TCN2* 776C>G polymorphism was determined using a Taqman allelic discrimination assay with primers and probes specific for the polymorphism and Taqman Universal Master Mix from Applied Biosystems (CA). No-template controls and duplicate DNA samples were included in the assays for quality control.

Peripheral Neurological Examination

Diagnosis of peripheral neuropathy was made by a board-certified neurologist from neurological history and a complete elementary neurological examination that included both lower limbs. The sensory exam included timed vibration with a 128 Hz tuning fork measured bilaterally at the distal phalanges of great toe. The distal vibration test was scored based on report of loss of vibration by the participant <10 s before the examiner (normal to mild), ≥ 10s (mild to moderate) or the inability of the patient to feel the maximum vibration of the tuning fork (severe to total). The predictive value and reproducibility of tuning fork test for peripheral neuropathy has been shown to be better than that of monofilament test.[25] In addition, subjects were evaluated for slow and fast proprioceptive sensation at the distal phalange of the great toe. Proprioceptive loss in either of these modalities was considered as evidence of large fiber neuropathy. Sensitivity to pin-prick, temperature and light touch were also determined and mapped to define a level of involvement typically in a distal to proximal pattern. Deep tendon reflexes at Achilles, patella, biceps and triceps were tested. Peripheral neuropathy was diagnosed as presence of a consistent pattern of large fiber polyneuropathy characterized by loss of either vibratory sense or proprioception. Typically these subjects had decreased or absent Achilles deep tendon reflexes. Neuropathy was also diagnosed if signs of small fiber loss characterized by loss of pain or

temperature sensation, numbness or dysesthesia were found on exam, typically in a distal to proximal pattern of loss. This pattern of sensory loss along with loss of deep tendon reflexes in the Achilles as well as patella was considered evidence of small fiber polyneuropathy. All the subjects were examined by the same neurologist.

Other Covariates

Folate and vitamin B_{12} intakes were determined from a validated semi-quantitative food frequency questionnaire.[26] Information on the other covariates used in this study were collected as follows: age, sex, race/ethnicity, education and smoking status (based on participant self-report), diabetes (based on blood glucose >126 mg/dL and reported use of diabetes medication), hypertension (based on systolic pressure greater than 140 mmHg or diastolic pressure greater than 90 mmHg and reported use of medications for hypertension) and body mass index (BMI, calculated as measured weight in kilograms divided by measured height in meters squared).

Statistical Analysis

Analysis of variance was used to describe the participant characteristics by genotype categories for continuous outcomes, and Pearson's chi-square test for categorical variables. The association between *TCN2* polymorphism and hypertension and diabetes was determined using logistic regression adjusting for age, sex, and BMI. Association of *TCN2* 776C>G polymorphism with plasma concentrations of vitamin B_{12} and logged total homocysteine was determined using analysis of covariance. Logistic regression was used to determine the odds ratio with 95% confidence interval (CI) of having peripheral neuropathy in the GG and CG genotypes compared to the reference genotype CC. In a secondary analysis to determine if folate intake modified the association of peripheral neuropathy with *TCN2* polymorphism we stratified the cohort based on folate intake exceeding twice the Recommended Dietary Allowance (>800 µg vs ≤800 µg/d of dietary folate equivalents) prior to calculating the association or odds ratio as described above. For all analyses, adjustments were made for the following covariates: age, sex, BMI,

race/ethnicity, education (as an indicator of socioeconomic status), serum creatinine concentration, smoking status, alcohol consumption, diabetes, hypertension and vitamin B_{12} intake. We adjusted for vitamin B_{12} intake in the analyses to distinguish the effect of the *TCN2* polymorphism from that of vitamin B_{12} intake on the outcomes. Additionally plasma concentration of folate and vitamin B_6 were used as covariates for plasma total homocysteine concentration, to identify the effect of *TCN2* polymorphism separate from that of these vitamins. Vitamin B_{12} intake and plasma concentrations of folate, pyridoxal 5'-phosphate and homocysteine were log-transformed prior to analyses since the distribution of these variables were skewed. Adjustments were made for multiple comparisons (Tukey's). There were 10 participants whose vitamin B_{12} intake was less than the recommended 2.4 μg/d. All the analyses were repeated after excluding these participants. All analyses were performed on SAS 9.3 (SAS Institute Inc, NC) and a two-sided $P < 0.05$ was considered significant.

Results

The prevalence of homozygosity for the variant allele G of the *TCN2* 776C>G polymorphism was 17.2% in this cohort (**Table 1**). Individuals with the GG and CG genotype had significantly lower plasma vitamin B_{12} compared to individuals with the CC genotype, even after adjusting for vitamin B_{12} intake ($P<0.02$, **Table 2**). Concentration of plasma total homocysteine was similar for all the genotypes after adjusting for covariates (Table 2).

Among the 171 participants who were assessed for peripheral neuropathy 75 individuals were diagnosed with peripheral neuropathy (**Table 3**). The odds of having peripheral neuropathy was 3.3 fold higher in individuals with GG genotypes compared to individuals with CC genotype (odds ratio 3.33; 95% CI: 1.15-9.64, Table 3). There was no difference between the CG genotype and the CC genotype in the odds for peripheral neuropathy (odds ratio 1.26; 95% CI: 0.56 – 2.81, Table 3).

Since high folate intake can worsen conditions associated with vitamin B_{12} insufficiency,[13] we determined if the association between the *TCN2* polymorphism and peripheral neuropathy was modified by folate intake in a secondary analysis. When folate intake was above twice the Recommended Dietary Allowance (800 μg), GG genotypes had 7 fold higher odds of neuropathy compared to CC genotype (odds ratio 6.9; 95% CI: 1.31-36.36, Table 3).There was no significant difference between the genotypes in odds ratio for peripheral neuropathy when folate intake was ≤800 μg (odds ratio 1.49; 95% CI: 0.18 – 12.33, Table 3). Limiting analyses to only those whose dietary intake of vitamin B_{12} met the recommended dietary allowance did not change the results for any of the outcomes studied.

Discussion

In this study we report the association of the 776C>G polymorphism in transcobalamin II gene *TCN2* with peripheral neuropathy in older adults with normal plasma concentration of vitamin B_{12}.

Peripheral neuropathy associated with vitamin B_{12} insufficiency is usually reversible with vitamin B_{12} treatment.[9] In our cohort, despite normal plasma concentration of vitamin B_{12}, the odds for development of peripheral neuropathy was 3.3 fold higher in the GG genotypes when compared to CC genotypes of the *TCN2* polymorphism. The association of *TCN2* polymorphism with peripheral neuropathy was independent of vitamin B_{12} intake.

High folate intake is associated with the worsening of the clinical conditions associated with vitamin B_{12} insufficiency in the elderly as indicated by the increased prevalence of anemia and cognitive impairment.[13,14,17] The mechanism behind this association is presently unkown. Recommended Dietary Allowance for folate is 400 μg/d of dietary folate equivalent. The odds ratio for neuropathy was significantly higher for the GG genotypes compared to CC genotypes when their folate intake was more than twice the recommended dietary allowance (>800 μg/d), but not when the folate intake was ≤ 800 μg/d. These results are supported by a recent study on vitamin B_{12} supplementation of elderly deficient in the vitamin, where the improvement of vitamin B_{12} status after vitamin B_{12} supplementation was impaired by high serum folate status.[27] Both natural folate and synthetic folic acid from fortified foods and supplements contributed to the total folate intake in our population. The mean folate intake of our cohort was 928.3 ± 576 μg dietary folate equivalents, which is similar to the mean folate intake of general U. S. population aged ≥51 years, as reported for the participants of the National Health and Nutrition Examination Survey (NHANES 2003-2006),[28] indicating that excess folate intake is widely prevalent among the older adults. Thus the results of our study are relevant to a large population

of older adults who are at increased risk for neurological impairment due to the *TCN2* polymorphism.

The prevalence of neuropathy was 43.9% among the study participants who were assessed for neuropathy. This is higher than the 23.8% that has been reported for those aged ≥60 years in the general US population in the NHANES (1999-2000).[29] One possible reason for this discrepancy could be the fact that NHANES population is healthier than our cohort, which is comprised of individuals who needed assistance for independent living. Despite the prevalence of peripheral neuropathy and its association with the *TCN2* polymorphism in the study population, macrocytic anemia, another condition that is frequently observed in vitamin B_{12} insufficiency was observed in only one participant. This finding is not unusual since previous studies have reported neurological disorders associated with low vitamin B_{12} in the absence of macrocytosis or anemia in the elderly.[9]

Individuals with GG and CG genotype had lower plasma concentration of vitamin B_{12} when compared to those with CC genotype even though the values were in the normal range in our cohort. This could be explained by the lower transcobalamin II protein concentration as well as its impaired binding of vitamin B_{12} in the GG and CG genotypes,[3,30] which may result in renal excretion of vitamin B_{12}. These results are different from that of previous studies that reported no difference between genotypes or that GG genotypes have higher vitamin B_{12} concentration in plasma.[4,5] We did not observe a relationship between *TCN2* polymorphism and plasma concentration of total homocysteine. There have been contradictory reports of higher homocysteine in individuals with GG genotypes[5,31] as well as a lack of genotype effect.[4,32]

A limitation of our study is the cross-sectional design of the cohort, which does not allow us to determine a cause-effect relationship. There were more women than men in the subset who were assessed for neuropathy, but women were at lower risk for peripheral neuropathy in our population as well as in others,[29] consequently a study with more men would only strengthen the

association between the polymorphism and neuropathy. Our study population is composed of individuals from different racial backgrounds, but they were predominantly Non-Hispanic Whites (61.4%) and Non-Hispanic African Americans (33.9%). While lower extremity diseases such as peripheral neuropathy is more prevalent among Non-Hispanic African Americans,[29] the *TCN2* polymorphism is less prevalent among this group when compared to Non-Hispanic Whites (Table 1). Hence the association between the *TCN2* polymorphism and peripheral neuropathy observed in this study was not driven by race. Diabetes increases the risk for peripheral neuropathy, but we didn't make a distinction between diabetic and non-diabetics in our analysis due to the small sample size. The prevalence of diabetes was similar among all the genotypes (Table 1) so the association of the *TCN2* polymorphism and peripheral neuropathy was not significantly affected by presence of diabetes.

Conclusions

In conclusion, our study shows that the *TCN2* 776C>G polymorphism is associated with increased risk for peripheral neuropathy in older adults despite adequate plasma vitamin B_{12} levels. The data also suggests that odds for peripheral neuropathy are higher for GG genotypes if they consume excess folate. Prevalence of peripheral neuropathy in the US population among those aged ≥60 years is 23.8% and is higher at 34.7% among those aged ≥80 years.[29] Intake of excess folate is also more prevalent among older adults in the US.[33] Our data suggests that the increased risk for neuropathy in older adults due to the *TCN2* polymorphism may be avoided by limiting the folate intake to be close to the Recommended Dietary Allowance and exercising caution with regards to consumption of folic acid supplements.

ARTICLE INFORMATION

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Author Contributions: Dr. Paul had full access to the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Paul

Acquisition, analysis or interpretation of data: Sawaengsri, Bergethon, Qiu, Scott, Jacques, Selhub, Paul

Drafting of the manuscript: Sawaengsri, Bergethon and Paul

Critical revision of the manuscript for important intellectual content: Sawaengsri, Bergethon, Qiu, Scott, Jacques, Selhub, Paul

Statistical analysis: Sawaengsri, Jacques

Obtaining funding: Selhub

Administrative, technical or material support: Paul, Selhub, Qiu

Study supervision: Paul, Selhub

Conflict of Interest disclosure: None reported

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conclusion, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the United States Department of Agriculture.

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Table 1. Characteristics of Study Participants by *TCN2* 776C>G Genotype[1]

Characteristic	Total (*n*=171)	CC (*n*=79)	CG (*n*=61)	GG (*n*=31)	P[2]
Age, mean (SD), y	73.5 (8.0)	73.2 (7.9)	73.8 (8.3)	73.8 (8.0)	0.90
Female, No. %	121 (70.8)	55 (69.6)	41 (67.2)	25 (80.7)	0.39
BMI, kg/m^2	31.9 (8.6)	32.6 (9.9)	31.7 (7.1)	30.5 (7.7)	0.50
Education, No. %					0.66
K-8th Grade	21 (12.3)	10 (12.7)	7 (11.5)	4 (12.9)	
9-11th Grade	19 (11.1)	11 (13.9)	6 (9.8)	2 (6.5)	
12th Grade/High School	47 (27.5)	25 (31.7)	17 (27.9)	5 (16.1)	
Any Undergraduate Education	70 (40.9)	28 (35.4)	26 (42.6)	16 (51.6)	
Any Graduate Education	14 (8.2)	5 (6.3)	5 (8.2)	4 (12.9)	
Race/Ethnicity, No. (%)					<0.001
American Indian /Alaskan Native	3 (1.8)	3 (3.8)	0 (0)	0 (0)	
Asian	3 (1.8)	1 (1.3)	0 (0)	2 (6.5)	
Non-Hispanic Black	58 (33.9)	39 (49.4)	16 (26.2)	3 (9.7)	
Hispanic	2 (1.2)	0 (0)	1 (1.6)	1 (3.2)	
Non-Hispanic White	105 (61.4)	36 (45.6)	44 (72.1)	25 (80.7)	
Current Smoker, No. (%)	39 (22.8)	18 (22.8)	14 (23.0)	7 (22.6)	1.00
Alcohol (g/day), mean (SD)	2.0 (5.4)	1.3 (4.3)	3.2 (7.2)	1.4 (3.2)	0.11
Vitamin B_{12} intake, mean (SD) (µg/d)	16.6 (16.6)	14.8 (16.8)	18.2 (16.0)	17.8 (17.5)	0.44
Folate intake, mean (SD) (µg DFE/d)	928.3 (575.9)	863.5 (616.0)	985.4 (543.0)	980.8 (531.5)	0.40
Plasma Metabolites, mean (SD)					
Folate, (ng/mL)[2]	14.1 (8.7)	13.7 (8.3)	13.9 (9.3)	15.2 (8.9)	0.71
Pyridoxal 5'-phosphate (ng/mL)[2]	19.7 (20.6)	18.9 (22.0)	20.5 (19.0)	20.3 (20.7)	0.89
Creatinine (mg/dL)[2]	0.9 (0.3)	0.9 (0.3)	0.9 (0.4)	0.8 (0.3)	0.68
Diabetes, No. (%)[3]	49 (28.7)	20 (25.3)	20 (32.8)	9 (29.0)	0.54
Hypertension, No. (%)[3]	142 (83.0)	68 (86.1)	53 (86.9)	21 (67.7)	0.21

[1]Between-genotype differences were analyzed with the use of Pearson's chi-square test for categorical variables and one-way ANOVA for continuous variables.

[2]To convert the values for folate from nanograms per milliliter to nanomoles per liter, multiply by 2.266. To convert values for pyridoxal 5'-phosphate from nanograms per milliliter to nanomoles per liter, multiply by 4.046. To convert the values for creatinine from milligrams/deciliter to micromoles per liter, multiply by 0.0884.

[3]Analysis of covariance adjusted for age, sex, race/ethnicity

Table 2. Concentrations of Plasma Metabolites by *TCN2* 776C>G Genotype

Metabolite	Total (*n*=171)	CC (*n*=79)	CG (*n*=61)	GG (*n*=31)	P
Vitamin B_{12} (pg/mL)[1]	496.1 (13.3)	539.8 (41.3)	456.1 (42.9)*	429.6 (45.9)**	<0.01
Homocysteine (μmol/L)[2]	10.7 (1.0)	9.9 (1.1)	10.1 (1.1)	10.7 (1.1)	0.61

[1] Mean (SE). To convert the values for vitamin B_{12} from picograms per milliliter to picomoles per liter, multiply by 0.7378. Analysis of covariance was used to determine the association of the *TCN2* polymorphism with plasma concentration of vitamin B_{12}. Adjustments were made for sex, age, BMI, education, alcohol intake, race/ethnicity, smoking status, vitamin B_{12} intake and plasma creatinine concentration as well as for multiple comparisons. *P<0.02 and **P= 0.01 compared to the CC genotype.

[2] Geometric mean (SE). Analysis of covariance was used to determine the association of the *TCN2* polymorphism with plasma concentration of homocysteine. Adjustments were made for sex, age, BMI, education, alcohol intake, race/ethnicity, smoking status, vitamin B_{12} intake, plasma concentrations of creatinine, folate and PLP as well as for multiple comparisons.

Table 3. Association between Peripheral Neuropathy and *TCN2* 776C>G Genotype[1]

	CC Genotype (Reference Group)		CG Genotype		GG Genotype (variant)	
	Cases/ Total	Odds Ratio	Cases/ Total	Odds Ratio (95% CI)	Cases/ Total	Odds Ratio (95% CI)
Overall	33/79	1	25/61	1.26 (0.56 – 2.81)	17/31	3.33 (1.15 – 9.64)*
Folate Intake:						
≤800 µg DFE	24/47	1	11/26	1.11 (0.31 – 3.93)	7/12	1.49 (0.18 – 12.33)
>800 µg DFE	9/32	1	14/35	1.30 (0.38 – 4.51)	10/19	6.90 (1.31 – 36.36) **

[1] Analysis of covariance adjusted for sex, age, BMI, education, alcohol intake, plasma creatinine concentration, race/ethnicity, smoking status, vitamin B_{12} intake, diagnosis of diabetes, and diagnosis of hypertension.

*P=0.03 **P=0.02

Chapter V

Summary, Discussion, Conclusion and Future Directions

Summary

Many people in the U.S. consume high amounts of folic acid, the synthetic form of folate used in supplements and fortified foods. However, consumption of folic acid greater than the capacity of DHFR to metabolize it leads to unmetabolized folic acid in plasma. Previous studies from our group showed that presence of unmetabolized folic acid in plasma, indicative of high folic acid intake, was associated with decreased natural killer (NK) cell cytotoxicity in women older than 60 years [1]. NK cells are innate immune cells important for defense against cancerous and virally infected cells and impairment in their activity increases disease risk. Thus, the finding that unmetabolized folic acid in plasma is associated with decreased NK cell cytotoxicity in older women may have negative implications for cancer development and susceptibility to infection in older populations. In addition to the effects of excess folic acid on immune function, high intakes of folic acid in conjunction with low vitamin B-12 status have led to increased clinical symptoms of vitamin B-12 deficiency such as cognitive decline and anemia [2-4]. The purpose of this thesis was to determine the negative effects of excess folic acid consumption.

In the first aim (Chapter II), we used an aged animal model fed 20x the RDA of folic acid (40mg/kg) to test the hypothesis that consumption of excess folic acid caused a reduction in NK cell cytotoxicity. We found that aged female mice fed a high folic acid diet had reduced NK cell cytotoxicity in spleen, lower mature cytotoxic /naïve NK cell ratio, and decreased production of lipopolysaccharide stimulated IL-10 secretion compared to mice on control diet. The difference in NK cell cytotoxicity between dietary groups was abolished when splenocytes were supplemented with exogenous IL-10 prior to assessment of NK cell cytotoxicity, suggesting that

reduced NK cell cytotoxicity in the high folic acid group was at least partially due to reduced IL-10 production.

The second aim (Chapter III) used the same animal model to test the hypothesis that excess folic acid would increase susceptibility and severity to influenza infection. We found that aged female mice fed a high folic acid had increased viral titer 2 days after infection and reduced cytokine gene expression in lung cells 5 days after infection. The implications of these findings are that aged female mice fed a high folic acid had lower NK cell cytotoxicity, as reported in in the first aim, leading to an inability to effectively clear the viral infection. In addition, NK cells secret cytokines that stimulate other immune cells to mount a response to the viral infection. Impaired NK cell activity may lead to impaired immune cell activation and decreased inflammatory cytokine secretion which may also lead to impaired viral clearance. However, there were no differences in weight loss, lipopolysaccharide stimulated cytokine secretion or cytokine protein in lung supernatant. Further evidence is needed to verify that excess folic acid led to increased severity of influenza infection in an aged mouse model.

In the third aim (Chapter IV), we wanted to determine if the vitamin B-12 transporter transcobalamin II (TCN2) polymorphism was associated with peripheral neuropathy, a symptom of vitamin B-12 deficiency. In addition, determine if excess folic acid intake would increase the association. We used cross sectional data from the Nutrition, Aging, and Memory in Elders (NAME) Study which consisted of homebound elders with sufficient vitamin B-12 status. We found that that homozygosity for the TCN2 TCN2 polymorphism was associated with 3 fold higher odds for peripheral neuropathy in elderly despite normal vitamin B12 status. These odds were increased to 7 fold if their folate intake was in excess of twice the recommended dietary

allowance, supporting the hypothesis that excess folic acid intake may be harmful in case of low vitamin B-12 status.

Overall, these results demonstrate that excess folic acid may be harmful to the aging population and that further studies are needed to elucidate the mechanisms.

Limitations

The limitations to the first two aims are that they were conducted in an aged mouse model since it is unethical to conduct such research in humans. This limits translatability of the findings to humans. However, the original hypothesis linking unmetabolized folic acid in plasma with reduced NK cell cytotoxicity was found in postmenopausal women suggesting a high probability of translating the work of the first two aims to humans. An additional limitation to the animal model used in both aim 1 and 2 is that the high folic acid diet groups were fed 20x the RDA of folic acid. Although some interventions have used this concentration of folic acid, very few people in the population consume this much folic acid. However, this amount was used in order to overcome the extremely higher DHFR activity in rodents, which can be up to 56 times higher than human DHFR [5]. A high dose is needed to ensure that the folic acid ingested was more than the rodent DHFR could metabolize so there would be unmetabolized folic acid in the plasma. It was uncertain if the effects seen in Troen's study were due to high folic acid intake or presence of unmetabolized folic acid in plasma so we wanted to ensure the mice on the high folic acid diet would have unmetabolized folic acid in the plasma[1].

A limitation to the second aim is that many mice died during the aging process, and after influenza infection, PBS sham control mice also lost weight during the experiment suggesting a

confounding effect of the study. In addition, the findings were limited to 5 days post infection due to loss of mice during the aging process leading to insufficient sample size for a day 7 post infection time point measurement. Thus duration of influenza infection could not be determined for the high folic acid and control diet groups since both groups had detectable viral titer by day 5. All lung samples were used for isolation of lymphocytes for NK cell cytotoxicity assay, NK subset analysis, ex vivo cytokine secretion assay, and folate content analysis leaving no samples for histology to assess pathology of lung and no sample to determine lung concentration of cytokines. We address this issue by determining gene expression of inflammatory cytokines in lung tissue.

The limitation of the third aim is that it is a cross sectional study with limited sample size. However, it is the first study to address whether the TCN2 776 C>G polymorphism leads to clinical outcomes of vitamin B-12 deficiency in people with normal vitamin B-12 status.

Appendix Sections

The appendix section of this dissertation included work conducted during the thesis that was ancillary to the three aims proposed. In the first section of the appendix, we showed the difference between young (3 months) and aged (16 months) mice when fed a high folic acid diet (20x RDA, 40mg/kg) or a control diet (1x RDA of folic acid, 2mg/kg). There were no differences in immune outcome in young mice fed a high folic acid and a control diet, highlighting the significance of age in the effects of folic acid on NK cell cytotoxicity.

The second section of the appendix used the same cohort and analysis as aim 3 except with different outcomes. We found that anemia, depression score, memory, executive function, and attention were not different between the TCN2 776C->G polymorphism irrespective of

folate intake. Mini Mental State Examination scores were slightly lower in GG genotypes (23.9±6.6 when compared to CG genotypes (24.9±9.37, P=0.02) but not CC genotypes (24.4±9.75, P=0.48), this association was not affected by folate intake.

Conclusions

Overall the work described in this thesis strongly suggests:

1. Excess intake of folic acid leads to decreased NK cell cytotoxicity in an aged mouse model.
2. Excess intake of folic acid leads to increased viral titer during influenza infection in an aged mouse model.
3. TCN2 TCN2 polymorphism is associated with increased odds for peripheral neuropathy and intakes of folate greater than 2x the RDA worsens these associations.

Future Directions

We plan to pursue the findings from the influenza animal model to determine conclusively if excess folic acid intake extends the recovery period from an infection since that time point was not captured in the present thesis. In addition, lung pathology will be collected to determine if excess folic acid intake led to decreased lung tissue damage since inflammatory cytokines were decreased in Chapter III.

We also plan to follow up on the findings from aim 3 to study the effects of excess folic acid in low vitamin B-12 conditions to determine the mechanism behind this interaction. A grant using the data from aim 3 is currently being submitted requesting funding to study the effects of excess folic acid in vitamin B-12 deficiency in the *C. elegans* model.

In conclusion, the overall finding that excess folic acid intake in the elderly can lead to decreased immune function and increased clinical symptoms of vitamin B-12 deficiency warrants further study.

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Chapter VI

Appendix

Appendix – Part I: High Folic Acid Intake Reduces Natural Killer Cell Cytotoxicity in Aged but Not Young Mice ☆

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Running title: High folic acid diet & natural killer cell cytotoxicity

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Keywords: Folic acid, folate, natural killer cell cytotoxicity, interleukin-10, aged mice.

Abstract

Unmetabolized plasma folic acid is associated with reduced natural killer (NK) cell cytotoxicity in post-menopausal women ≥ 60 years, which may have important implications for carcinogenesis and infection. Our objective was to establish a mouse model to explore the mechanism behind reduced NK cell activity associated with high folic acid intake.

Aged (16 months) and young (3 months) female C57B/6 mice were fed a control diet with 1xRDA of folic acid (2mg/kg) or a high folic acid diet with 20xRDA of folic acid (40mg/kg) for 3 months.

High folic acid diet in aged mice resulted in reduced NK cytotoxicity for effector to target cell ratios 25:1 (15% decrease P=0.0263), and 12.5:1 (24% decrease P=0.0235) compared to aged mice on control diet. Aged mice fed a high folic acid diet had decreased ratio of mature to naïve NK cells and reduced interleukin-10 production after stimulation with lipopolysaccharide for 24 hours. In young mice, there were no differences in NK cytotoxicity, percent of NK cells, or NK cell subset distribution or ratios between the two diet groups. However, young mice fed a high folic acid diet had significantly less production of tumor necrosis factor alpha (TNF-α) after stimulation with lipopolysaccharide for 24 hours.

This data suggests that NK cytotoxicity may be affected in aged mice consuming a high folic acid diet by reduced percentages of NK cells but not in young mice. In future studies we can study the downstream effect on high folic acid diet on immune response to infection in aged mice.

Keywords: Folic acid, folate, natural killer cell cytotoxicity, immune function

1. Introduction

Folate is a vitamin essential for health throughout life so the United States along with many other countries mandated folic acid fortification in flour and grain products. However, in addition to consumption of fortified foods and cold breakfast cereals which may already contain 100% the recommended dietary allowance (RDA) of folic acid, approximately 39% of the U.S. population consumes multivitamin pills containing folic acid, with the majority of consumers being women and people over the age of 60 [1, 2] . Approximately 35% of the US population consumes folic acid containing dietary supplements and 5% exceed the tolerable upper intake level of 1000 μg/d for folic acid [1]. The recommended daily allowance (RDA) for folate is 400 μg dietary folate equivalents, which is equal to 400 μg natural folate or 240 μg folic acid [2, 3]. Folic acid is the synthetic form of folate used in supplements and food fortification due to its stability. It requires reduction to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR) [3]. In humans, DHFR is a slow enzyme with poor affinity for folic acid [4]. In addition, genetic variations in DHFR also influence the ability of an individual to metabolize folic acid [5]. Consumption of folic acid in excess of the metabolic capacity of the body leads to the appearance of unmetabolized folic acid in plasma [4, 5].

We have previously shown that the presence of unmetabolized folic acid in plasma, indicative of folic acid intake beyond the metabolic capacity of the body, is associated with reduced natural killer (NK) cell cytotoxicity in postmenopausal women aged 60-75 years but not in women aged 50-59 years old [6]. These findings suggest that aging may modify the association between presence of unmetabolized folic acid in plasma and reduced NK cytotoxicity. NK cells are cytotoxic lymphocytes that are part of the innate immune system and are important for surveillance and defense against virus-infected and cancer cells. They bind to target cells and perforate the cell membrane by secreting pore-forming protein perforin and trigger apoptosis by secreting granzymes [7]. Low NK cytotoxicity is associated with increased

risk for cancer in humans [8]. Hence it is important to determine if there is a causal relationship between high folic acid intake and NK cytotoxicity and if age modifies this relationship.

2. Materials and Methods

2.1. Animals and diets.

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and conducted according to the Guide for the Care and Use of Laboratory Animals (1996). Two to four month old and sixteen-month-aged female C57BL/6 mice were purchased from National Institute of Aging colonies at Charles River Breeding Laboratories (Wilmington, MA). The mice were housed on a 12 hour light/dark cycle and provided free access to the diets throughout the experiment. Twelve mice per diet group per age group were maintained on an AIN-93M [9] based diet (Harlan-Teklad, Madison, WI) with the American Institute of Nutrition recommended daily allowance of 2 mg/kg diet folic acid (control diet, 1x RDA) or 40 mg/kg diet folic acid (high folic acid diet, 20x RDA) for 3 months. The dosage of folic acid in the high folic acid diet was determined based on a previous study that investigated the effect of high folic acid diet in mice [10]. In addition, equivalent dosage of folic acid is used in many intervention trials of age-related chronic illnesses [11, 12].

2.2. Spleen cell isolation.

Mice were euthanized by CO_2 asphyxiation followed by exsanguination through cardiac puncture, and blood was used for plasma isolation. Spleen was removed aseptically and single-cell suspensions were prepared as previously described [13]. Spleens were placed in sterile RPMI 1640 medium (Lonza, MA) which was supplemented with 5% heat inactivated fetal bovine serum, 25 mmol/L HEPES, 2 mmol/L glutamine, 100 kU/L penicillin, and 100 mg/L streptomycin (Life Technologies, CA) and disrupted between two sterile frosted glass slides. The

cells were collected by centrifugation at 300G for 10 min and resuspended in red blood cell lysis buffer (Sigma, MO) to lyse red blood cells. After washing with phosphate buffered saline and removing cell debris by filtering through 40 μM nylon cell strainer (BD Pharmingen), splenocytes were counted using an Accuri C6 flow cytometer (BD Accuri Cytometers, MI) and resuspended in appropriate media for the assays.

2.3. Preparation of target cells.

YAC-1 cells, a murine T- lymphoma cell line sensitive to NK cell killing, were grown in complete RPMI 1640 medium prepared as above and maintained at 37°C in 5% CO_2. For the 5 days before the assay, cells were subcultured every 24 h to ensure that they were in the log phase. The cells used for the assay were stained with 10 nM of carboxyfluorescein succinimidyl ester (CFSE, eBioscience, CA) for 10 minutes at room temperature in the dark, washed twice in phosphate buffered saline with 2% heat-inactivated fetal bovine serum and resuspended to a final concentration of 10^6 cells/mL in RPMI 1640 medium.

2.4. NK cytotoxicity assay.

The flow cytometric assay described by McGinnes et al [14] was used with modifications as described by Cao et al. [15]. All reagents were purchased from eBioscience. Splenocytes (10^7/tube) were stained with allophycocyanin (APC)-conjugated anti-CD3 antibody and phycoerythrin (PE)-conjugated anti-NK1.1 antibody to identify NK cells ($CD3^-$ $NK1.1^+$). Stained splenocytes were then incubated with CFSE-labelled YAC-1 cells (target cells) at effector-to-target (E:T) cell ratios of 100:1, 50:1, 25:1, and 12.5:1 for 3 h at 37C with 5% CO_2. After the incubation was complete, 7-amino-actinomycin D (7-AAD) was added to each tube to a final concentration of 1.11 μg/mL. Dead YAC-1 cells, which were identified as 7-AAD and CFSE double positive cells, were counted using an Accuri C6 flow cytometer and acquired data were analyzed with FlowJo 7.6 software (Treestar Inc., OR). CFSE stained YAC-1 cells incubated in the absence of splenocytes were used to determine spontaneously dead target cells.

Percent specific target cell death (cytotoxicity) was then expressed as: 100 x {[dead YAC-1 Cells (%) – spontaneously dead YAC-1 Cells (%)]/ [100 – spontaneously dead YAC-1 target cells (%)]}. Number of live target cells were identified as CFSE positive and 7-AAD negative cells.

2.5. Immunophenotyping.

Splenocyte populations were determined using flow cytometry. Splenocytes were blocked using anti-CD16/32 antibodies and then stained using the following fluorochrome-conjugated antibodies: PE or APC-anti-CD3, APC-anti-CD4, fluorescein isothiocyanate (FITC) anti-CD8 and PE-anti-NK1.1 (eBioscience), to identify all T cells ($CD3^+$), T-helper cells ($CD3^+$ $CD4^+$), cytotoxic T cells ($CD3^+$ $CD8^+$), NK cells ($CD3^-$ $NK1.1^+$) and natural killer T (NKT) cells which have the properties of both T cells and NK cells ($CD3^+$ $NK1.1^+$). The stained cells were then analyzed using Accuri C6 flow cytometer and acquired data analyzed with FlowJo 7.6 to determine the immune cell phenotype.

Natural killer cell subsets were identified as previously described [16] using the following fluorochrome conjugated antibodies: APC-anti-CD3, PE-anti-NK1.1, FITC-anti-CD27 and PerCP-Cy5.5-anti-CD11b. All staining reactions included isotype controls to detect non-specific background signals. The antibodies and the respective isotype controls were purchased from eBioscience. The stained cells were analyzed using an Accuri C6 flow cytometer and the data were analyzed with FlowJo 7.6. Since a small portion of spleen was removed for folate analysis and the remaining spleen was immediately aseptically transferred to cell culture medium without weighing, the absolute numbers of the various cell types in spleen were not determined. Populations of various cell types were instead determined as percentage of total splenocytes.

2.6. Cytokine production.

Splenocytes in complete RPMI 1640 medium were stimulated in separate reactions with bacterial lipopolysaccharide (LPS, 1μg/mL) for 24 h, concanavalin A (Con A) (1.5μg/mL), or

anti-CD28 antibody (1 μg/mL) and anti-CD3 antibody (5μg/mL) coated on cell culture plates for 48 hours . At the end of the incubation, cell-free supernatants were collected and stored at -80°C until analysis. The concentration of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and interferon (IFN)-γ in the supernatants from LPS stimulated cells, and the concentration of IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF- α in the supernatants of Con A or anti-CD3and anti-CD28 stimulated cells were determined using the mouse cytometric bead array kit according to the manufacturer's instructions (CBA; BD Biosciences, CA). The fluorescence signals associated with cytokine-bead complex were acquired using Accuri C6 flow cytometer, and data were analyzed using FCAP Array™ Software 3.0.1 (CBA; BD Biosciences).

2.7. Stimulation of NK cytotoxicity by IL-10.

Splenocytes ($6x10^6$/well) were incubated with recombinant mouse IL-10 (eBioscience) at a final concentration of 15 ng/mL for 15 hours before measuring NK cytotoxicity as described above for the E:T ratios 25:1 and 12.5:1.

2.8. Folate analysis.

Total folate from spleen was determined using a microbial assay with *Lactobacillus casei* [17]. Protein concentration of the spleen extract used for folate assay was determined by Bradford method [18] using Bio-Rad protein assay reagent (Bio-Rad, CA). Folate forms in non-fasting plasma samples were analyzed by HPLC-affinity chromatography with electrochemical detection [19].

2.9. Statistical analyses.

Results are expressed as means ± SEM. One way ANOVA was used for the effect of diet on NK cell cytotoxicity adjusted for day of experiment. Student's t-test was used for all other

continuous outcomes. Significance was determined at $P<0.05$. Statistical analysis was performed using SAS 9.3 (NC).

3. Results

3.1. Effect of high folic acid diet on tissue and plasma folate content.

Both young and aged animals on both control and high folic acid diets gained weight during the 3 months on experimental diets, but there was no difference between the two diet groups in weight gain (data not shown). Analysis of non-fasting plasma showed that both young and aged mice on high folic acid diet had significantly higher concentration of unmetabolized folic acid, methyl tetrahydrofolate and formylated tetrahydrofolate when compared to mice on the control diet (Fig. 1A). Since the NK cell cytotoxicity was determined using splenocytes, we also determined the folate concentration of spleen. Young and aged mice fed a high folic acid diet had a higher concentration of total folate in spleen when compared to those on the control diet (22.45 ± 1.05 vs 15.22 ± 1.07 ng folate/mg protein in young mice, and 18.21 ± 0.76 vs 12.38 ± 0.96 ng folate /mg protein in aged mice, $P<0.01$ for both age groups, Fig. 1B).

3.2. Effect of high folic acid diet on NK cell cytotoxicity.

The mean NK cell cytotoxicity of aged mice fed a high folic acid diet was significantly lower than that of the mice fed the control diet at effector to target cell ratios of 25:1 ($P=0.03$) and 12.5:1 ($P=0.04$) by 14% and 23% respectively (Fig. 2A). We determined if the difference in NK cell cytotoxicity of the mice on the two diets was due to differences in the percentage of NK cells. Since the NK cell cytotoxicity assay measures the activity of both NK and NKT cells, we also measured the percentage of NKT cells in the spleen. While the percentage of NK cells in the spleen was lower in the high folic acid group compared to the control group, the difference was not statistically significant ($P=0.15$) (Fig. 3). The percentage of NKT cells in spleen was similar

in both diet groups (Fig. 3). The percentages of T helper cells and cytotoxic T-cells in total spleen cells were also similar in both diet groups (Supplementary Fig. 1).

However, there were no differences in NK cell cytotoxicity in young mice fed a high folic acid diet compared to a control diet (Fig. 2A). There were no differences in percent NK, NKT cells, T helper, or T cytotoxic cells (Fig. 3, Supplementary Fig. 1). Previous research has shown that young mice have enhanced NK cell cytotoxicity compared to aged mice [20]. Since young mice had lower NK cell cytotoxicity compared to aged mice in this study when the outcome measured was percent dead target cells, there is a potential that the young mice may have been very efficient at killing to the point where dead target cells are fragmented and no longer register on the flow cytometry. Thus, we also determined number of live target cells after conducting the NK cell cytotoxicity. We found that young mice had less number of live cells compared to aged mice, indicative of increased killing activity, but there was no effect of diet (Fig. 2B).

3.3. Effect of high folic acid diet on NK cell subsets

We determined the population of NK subsets in both diet groups based on the presence of the surface markers CD11b and CD27 [21]. Double negative (DN, $CD11b^-$ $CD27^-$) cells are NK cell precursors without effector function, $CD11b^-$ $CD27^+$ (R1 subset) are naïve and have reduced effector functions, $CD11b^+$ $CD27^+$ (R2 subset) are considered mature NK cells capable of killing target cells and secreting cytokines, and $CD11b^+$ $CD27^-$ (R3 subset) are terminally differentiated, mature NK cells that predominantly secretes cytokines [16, 21-23]. In aged mice fed a high folic acid diet there was a trend for a lower percentage of mature R2 NK cells (P=0.08) and higher percentage of naïve R1 cells (P=0.08) when compared to the control group (Fig. 4A). The percentage of DN and R3 NK cells was similar in both diet groups for aged mice (Fig. 4A). The ratio of R2 to R1 NK cells (P =0.03), but not that of R3 to R1 was significantly lower in the high folic acid fed aged mice when compared to those on the control diet (Fig. 4B). There were no

differences in NK subsets or the ratio of mature to naïve NK cells between the diet groups in young mice.

3.4. Effect of high folic acid diet on cytokine production by splenocytes

Since the percentage of NK cells and activation are dependent on cytokines we determined the production of cytokines (IL-10, IFN-γ, IL-6, TNF-α) and chemokine (MCP-1) by splenocytes after LPS stimulation. LPS stimulated a wide array of cell types in the splenocytes. The production of IL-10 by splenocytes from the aged mice in the high folic acid group were lower than that of the control group ($P<0.05$) (Fig. 5). The production of other cytokines was not different between the two diet groups in aged mice. The production of TNF-α in young mice fed a high folic acid diet was significantly lower than young mice fed a control diet ($P<0.05$) (Fig. 5). There were no differences in the production of other cytokines in young mice.

To determine if the low IL-10 production contributes to the reduced NK cell cytotoxicity in the high folic acid fed mice we stimulated the splenocytes of aged and young mice with recombinant IL-10 prior to the NK cell cytotoxicity assay. After stimulation with IL-10, the NK cell cytotoxicity of the high folic acid group was similar to that of the control group for both age groups (Fig. 6). When only the T cells among the splenocytes were specifically activated with Con A or antibodies against CD3 and CD28, there was no difference in the production of cytokines IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF- α between the 2 diet groups for both age groups (Supplementary Fig. 2A and 2B).

4. Discussion

In this study, we determined if high folic acid affected young and aged mice differentially and established a causal effect of high folic acid intake on lower NK cell cytotoxicity in aged mice. In aged mice, a high folic acid diet increased the concentration of total folate in spleen tissue, as well as the concentrations of unmetabolized folic acid, methyl tetrahydrofolate and

formyl tetrahydrofolate in plasma when compared to control diet (Fig. 1). We measured the plasma folate concentrations under non-fasting rather than fasting conditions to determine the extent to which the mice are exposed to the various folate forms after consuming the experimental diet. Intake of a high folic acid diet resulted in up to 23% lower NK cell cytotoxicity in splenocytes of aged mice when compared to consumption of recommended dose of folic acid ($P < 0.04$) (Fig. 2). We found a significant reduction in the ratio of mature cytotoxic to naïve NK cells in aged mice fed a high folic acid diet compared to the control diet ($P<0.03$) (Fig. 3) and reduced IL-10 secretion after LPS stimulation. IL-10 is a pleiotropic cytokine which has immunosuppressive functions but at the same time increases cytotoxicity of NK cells [24, 25]. A role for IL-10 in the lower NK cytotoxicity of the high folic acid fed mice is suggested by the fact that, the difference in NK cell cytotoxicity between the dietary groups was abolished when the splenocytes were stimulated by exogenous IL-10 prior to the NK cell assay (Fig. 6).

However, there were no diet effects on NK cell cytotoxicity, percent NK cell in spleens, or distribution of mature and naïve NK cells in young mice despite seeing significantly increased plasma folate forms and total spleen folate concentrations (Fig. 1A and 1B). However, young mice fed a high folic acid diet had significantly lower TNF-α expression after 24 hour stimulation with LPS, suggesting a possible anti-inflammatory effect of high folic acid feeding in young mice (Fig. 5). These data suggest that high folic acid feeding may have differential effects on the immune system in different age groups.

This supports our earlier finding that folic acid intake in excess of the metabolic capacity of the body, as indicated by unmetabolized folic acid in plasma, is associated with lower NK cell cytotoxicity in postmenopausal women older than 60 years, but not in postmenopausal women ages 50-59 years old [6]. The concentrations of unmetabolized folic acid in aged mice fed a high folic acid diet were comparable to what was observed in post-prandial serum of human subjects after consumption of 1000 µg of folic acid from bread over a period of 6 hours in 200 µg doses [26].

There are no known mechanisms for why excess folic acid would affect young and aged mice differently. A potential mechanism is that there may be greater breakdown of folic acid in the elderly, leading to increased 6FP, which directly modifies the functions of immune cells. Pregnant women have increased breakdown of folic acid as determined by the concentration of its breakdown product, PABAglu, in urine [27], and this might be due to increased oxidative stress during pregnancy. Similarly, oxidative stress increases with age, which may lead to increased breakdown of folic acid to 6FP.

Studies suggest that the breakdown product, 6-formyl pterin (6FP), is a bioactive compound that can modify immune function [28-30]. 6FP incubated with human peripheral blood leukocytes induced intracellular generation of hydrogen peroxide (H_2O_2) by transferring an electron from NADH to oxygen [31]. Human T cells incubated with 6FP had increased internal reactive oxygen species (ROS), decreased cell proliferation, and impaired secretion of the cytokines interferon gamma (IFN-γ) and interleukin-2 (IL-2) through suppressed NF-κB-dependent transcription when stimulated with mitogenic lectin phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) [28]. A study by the same group showed that 6-FP inhibited nitric oxide synthesis in the murine macrophage cell line RAW 264.7 when stimulated with LPS and interferon gamma, suggesting an inhibition of inflammatory signaling by activated macrophages [30]. Activated macrophages can secrete cytokines that are known to activate NK cells [32], thus 6-FP may be able to inhibit NK cell activity through inhibition of activated macrophages.

Adequate folate nutrition is important for NK cell cytotoxicity and immune response [33, 34]. But high folic acid intake, mostly from consumption of supplements and heavily fortified foods can have negative outcomes. In recent years, many studies have reported association of high concentration of folate in plasma and multivitamin use with increased risk for breast cancer

in women [35-37]. Our study has provided the first evidence for linking excessive folic acid intake to impaired NK cell function. This observation is of major public health concern since the function of NK cells is immune surveillance against cancerous and pathogen infected cells. Low NK cytotoxicity has been associated with increased risk for cancer in a human study that followed-up the subjects for 11 years [8]. Hence, reduction of NK cell cytotoxicity due to high folic acid intake may increase the susceptibility to cancer and viral infections especially in the elderly who are already at increased risk for these diseases.

In summary, our data show that high folic acid intake reduces NK cell cytotoxicity in aged mice and this is possibly due to impairment of NK cell maturation. However, high folic acid did not affect young mice. Additional studies are needed to further determine the mechanism behind the effect of excess folic acid intake on NK cell cytotoxicity and establish its relevance to health outcomes in humans.

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Figure Legends

Fig. 1. Effect of a high folic acid diet on folate concentration in plasma and spleen of young and aged mice. (A) Folate forms in plasma as measured by HPLC. (B) Total folate concentration in spleen as measured by microbial assay using *Lactobacillus casei*. FA, unmetabolized folic acid; FF, formylated folates; MTHF, methyl tetrahydrofolate. Values are means ±SEM, *n*=10-11/group. * P<0.01.

Fig. 2. Effect of a high folic acid diet on NK cell cytotoxicity in young and aged mice. Splenocytes were isolated and incubated with YAC-1 target cells at 100:1, 50:1, 25:1, and 12.5:1 ratio of effector to target cells. (A) Dead target cells (YAC-1 cells, CFSE+, 7-AAD+) were quantified by flow cytometry. (B) Live target cells (YAC-1 cells, CFSE+, 7-AAD-) were quantified by flow cytometry. (Values are means ±SEM, *n*=10-11/group. * P<0.05

Fig. 3. Effect of a high folic acid diet on percent NK cells in splenocytes in young and aged mice. Percent of NK ($CD3^-$ $NK1.1^+$) or NKT ($CD3^+$ $NK1.1^+$) cells in total splenocytes quantified by flow cytometry. Values are means ±SEM, *n*=10-11/group. * P<0.05

Fig. 4. Effect of a high folic acid diet on NK cell subsets in splenocytes in young and aged mice. Splenocytes were isolated and stained with fluorescent dyes conjugated to anti-CD11b, CD27, and NK1.1 antibodies and quantified by flow cytometry. (A) Representative flow chart of NK cell subsets in spleen of a mouse. (B) $NK1.1^+$ cells were separated into the following subsets: DN, double negative NK cells (NK cell precursors, $CD11b^-$ $CD27^-$); R1, naïve NK cells ($CD11b^-$ $CD27^+$); R2, mature ready to kill NK cells (R2: $CD11b^+$ $CD27^+$) and R3, terminally mature predominantly cytokine producing NK cells ($CD11b^+$ $CD27^-$). (B) Ratio of R2 and R3 to R1 NK cells. Values are means ±SEM, *n*=9/group. * P<0.05

Fig. 5. Cytokine secretion by splenocytes stimulated with LPS for 24 hrs. Cytokines were measured with a cytometric bead array. Values are means ±SEM, *n*=10-11/group. * P<0.05

Fig. 6. Effect of a high folic acid diet on NK cell cytotoxicity after stimulation of splenocytes from young and aged mice with 15 ng/mL of recombinant mouse IL-10 for 15 hrs. Data shown for 25:1 effector to target cell ratio and is similar for the 12.5:1 effector to target cell ratio (not shown). Values are means ±SEM, *n*=10-11/group. * P<0.05

Supplementary Figures

Supplementary Figure 1. Effect of a high folic acid diet on T cell profile in splenocytes from young and aged mice. Percent T cells ($CD3^+$), T helper cells ($CD3^+$ $CD4^+$), and cytotoxic T cells ($CD3^+$ $CD8^+$) in the spleen. Values are means ±SEM, *n*=10-11/group. * P<0.05

Supplementary Figure 2. Effect of a high folic acid diet on cytokine secretion by splenocytes from young and aged mice stimulated with ConA or anti-CD3 plus anti-CD28. (A) Splenocytes were stimulated with ConA (1.5µg/mL) for 48 hrs or (B) with plate coated anti-CD3 (5µg/mL) plus soluble anti-CD28 (1µg/mL) for 48 hrs. Cytokines were measured with cytometric bead array. Values are means ±SEM, *n*=9-11/group. * P<0.05

Figure 1

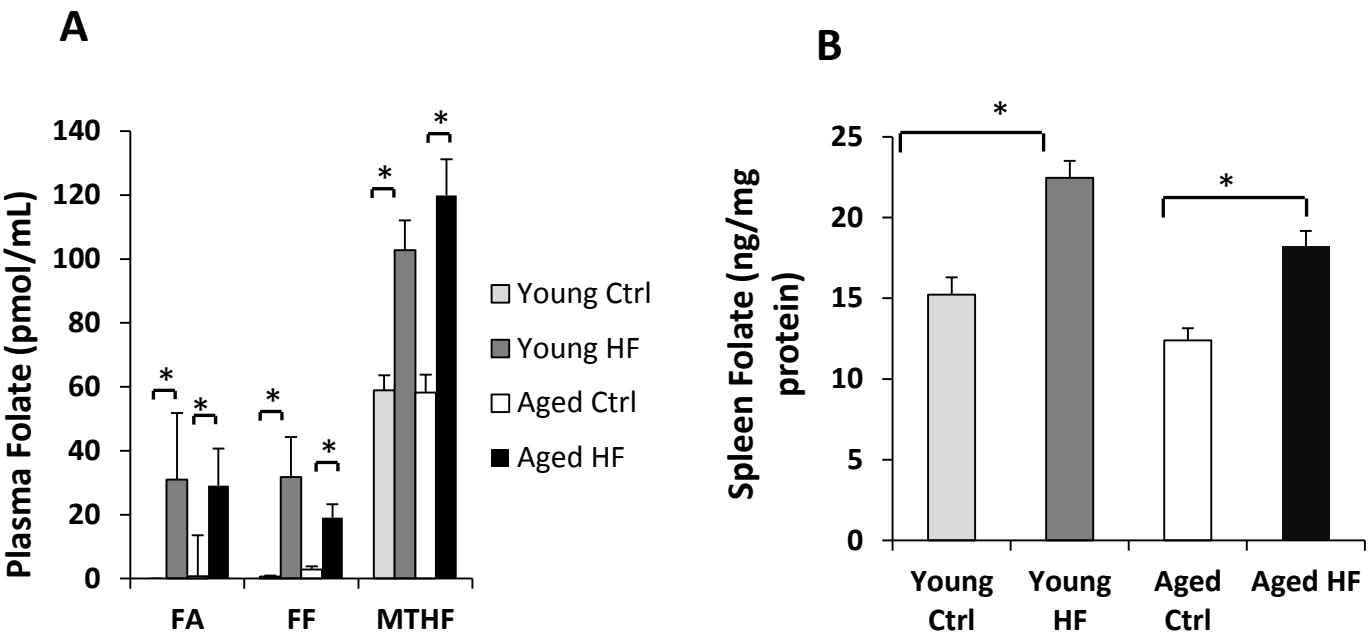
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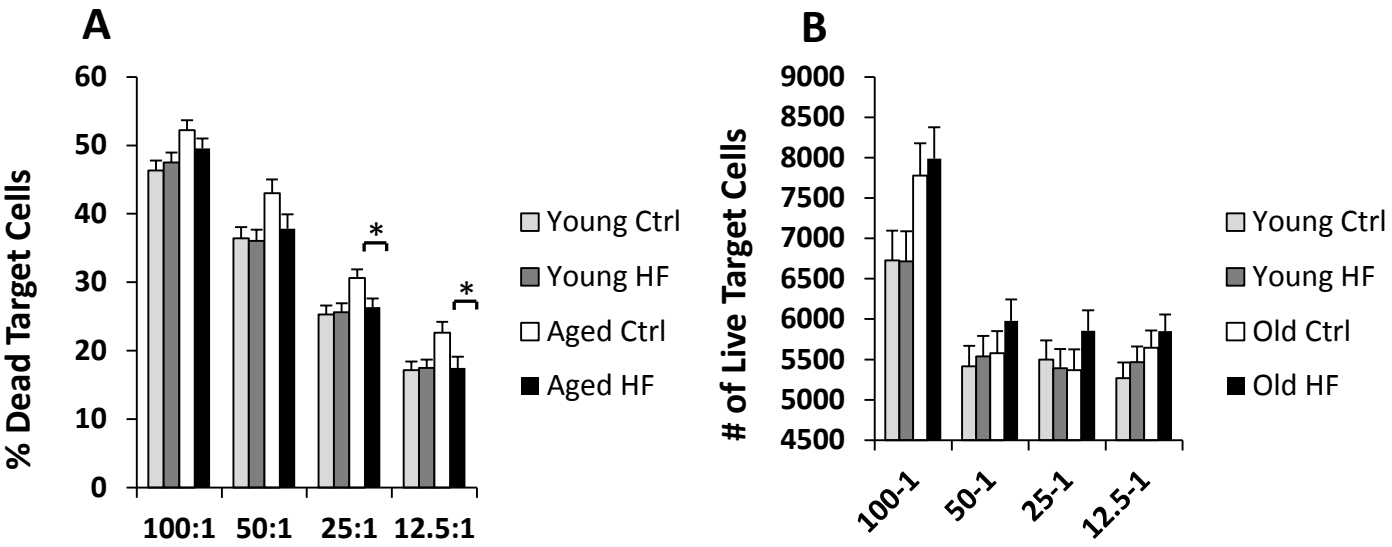
Figure 2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Old HF

Figure 3

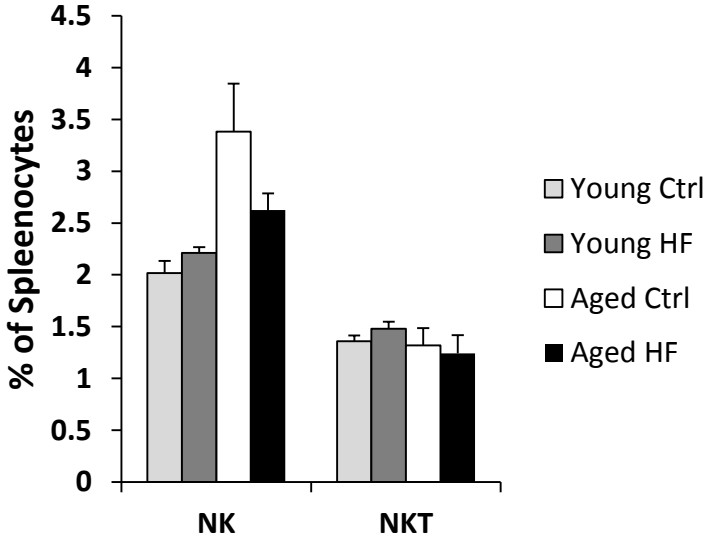

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
% of Spleenocytes
NK
NKT
Young Ctrl
Young HF
Aged Ctrl
Aged HF

Figure 4

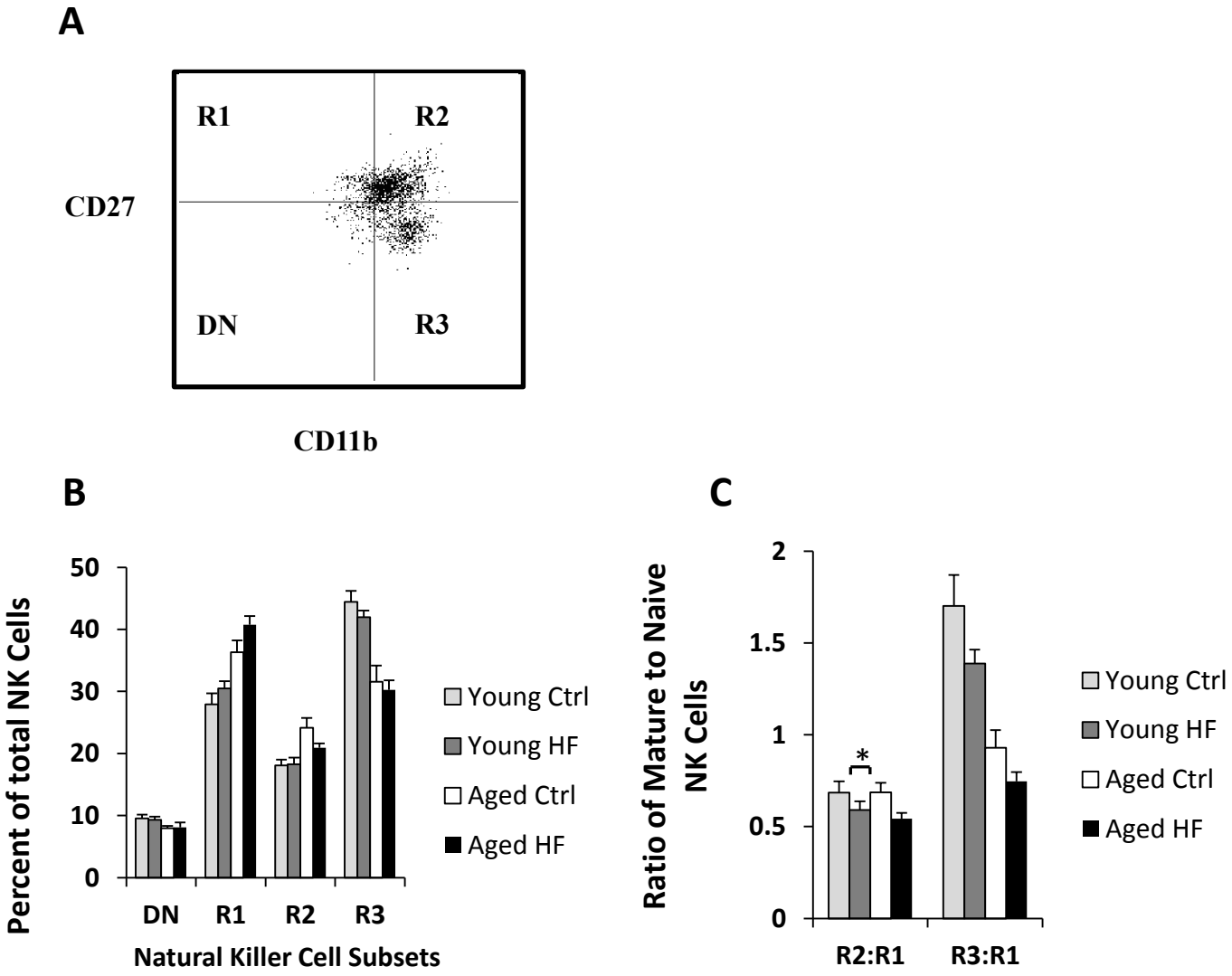

A
R1
R2
CD27
DN
R3
CD11b
B
Percent of total NK Cells
50
40
30
20
10
0
DN
R1
R2
R3
Natural Killer Cell Subsets
Young Ctrl
Young HF
Aged Ctrl
Aged HF
C
Ratio of Mature to Naive NK Cells
2
1.5
1
0.5
0
*
R2:R1
R3:R1
Young Ctrl
Young HF
Aged Ctrl
Aged HF

Figure 5

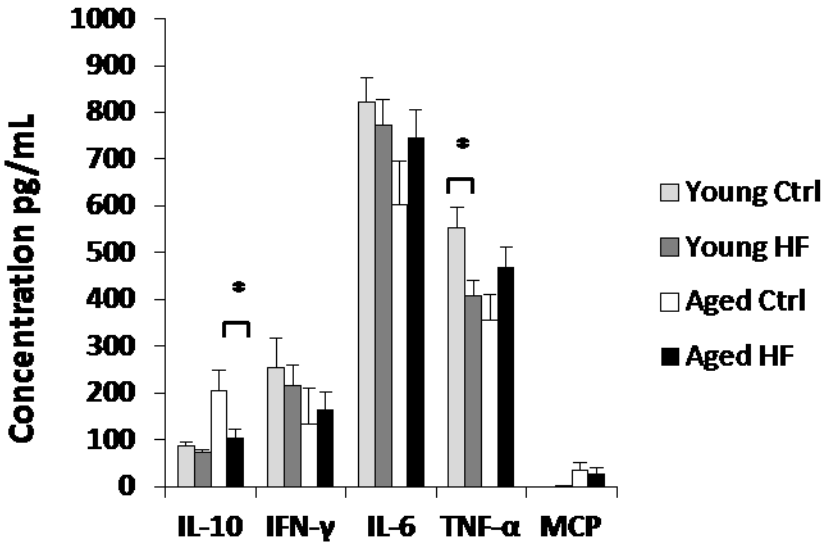
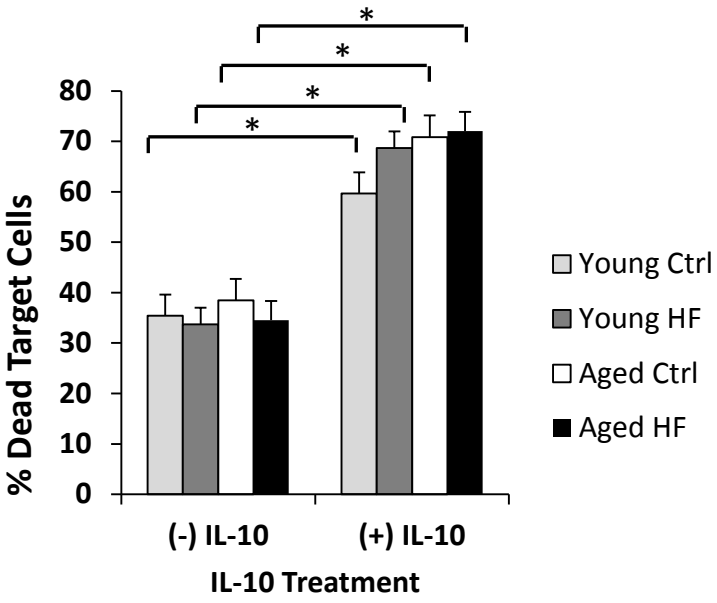
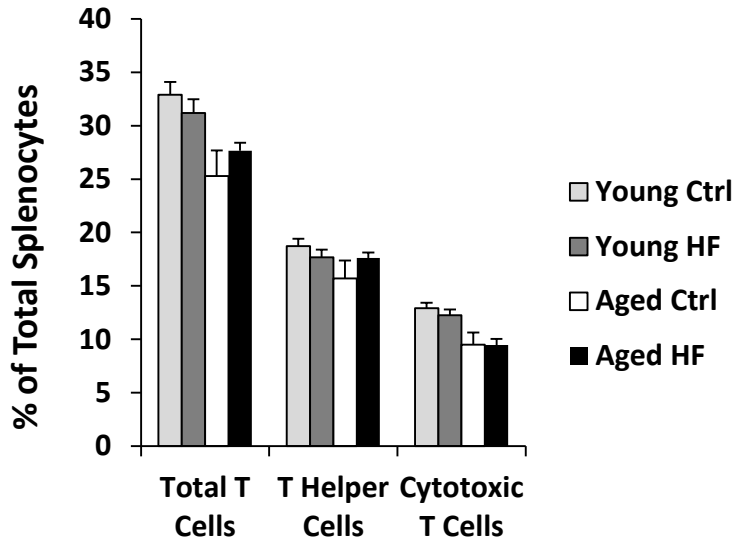

Concentration pg/mL
1000
900
800
700
600
500
400
300
200
100
0
*
*
IL-10
IFN-γ
IL-6
TNF-α
MCP
Young Ctrl
Young HF
Aged Ctrl
Aged HF

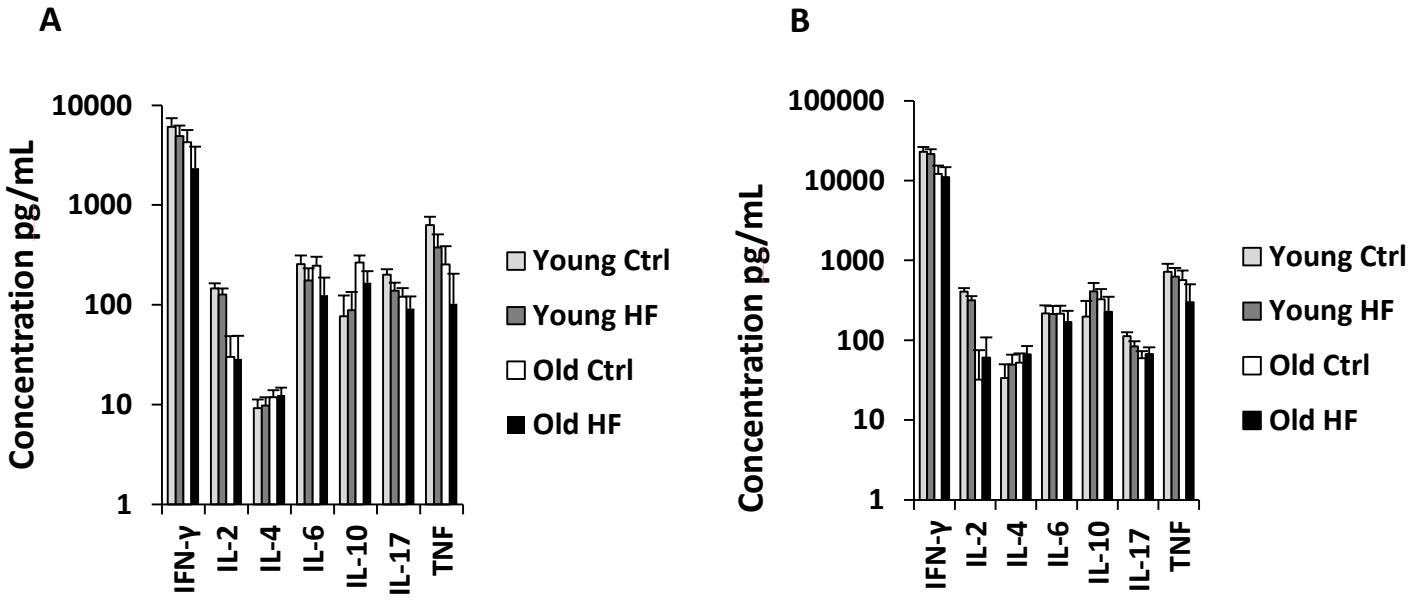
Figure 6


*
*
*
*
80
70
60
50
40
30
20
10
0
% Dead Target Cells
(-) IL-10
(+) IL-10
IL-10 Treatment
Young Ctrl
Young HF
Aged Ctrl
Aged HF

Supplementary Figure 1



Supplementary Figure 2



Appendix – Part II: Transcobalamin II 776C>G Polymorphism and Anemia, Depression and Cognitive Function in the Elderly

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ABSTRACT

BACKGROUND

The 776C>G polymorphism of the vitamin B_{12} transport protein transcobalamin II gene (*TCN2*, rs1801198, Pro259Arg) is associated with lower holotranscobalamin concentration in plasma. This can reduce the availability of vitamin B_{12} to tissues, even when vitamin B_{12} intake is adequate. Clinical outcomes associated with vitamin B_{12} insufficiency are worsened by high folate intake.

METHODS

We determined the effect of *TCN2* 776C>G polymorphism and folate intake on anemia, depression and cognitive function (n=574) in a population of homebound elders aged ≥60 y with normal plasma concentration of vitamin B_{12}.

RESULTS

Anemia, depression score, memory, executive function, and attention were not different between the genotypes irrespective of folate intake. Mini Mental State Examination scores were slightly lower in GG genotypes (23.9±6.6 when compared to CG genotypes (24.9±9.37, P=0.02) but not CC genotypes (24.4±9.75, P=0.48), this association was not affected by folate intake.

CONCLUSION

TCN2 776C>G polymorphism was not associated with anemia, depression score, memory, executive function, and attention in elderly with normal vitamin B_{12} status, and folate intake in excess of twice the recommended dietary allowance did not modify these outcomes.

INTRODUCTION

Transcobalamin II is a vitamin B_{12} binding protein that transports the vitamin from ileum to the tissues.[1] The 776C>G single nucleotide polymorphism in the gene for transcobalamin, *TCN2*, (rs1801198) results in a Pro259Arg substitution in the transcobalamin II protein.[2] This polymorphism affects binding of vitamin B_{12} by transcoblamin II and is associated with lower concentration of holotranscobalamin (vitamin B_{12}-bound transcobalamin) in plasma and can thus affect the availability of vitamin B_{12} to the cells. [3, 4] The plasma concentrations of homocysteine and methylmalonic acid are used as functional biomarkers for vitamin B_{12} status. The GG genotype of the 776C>G polymorphism of *TCN2* is associated with higher concentration of homocysteine in individuals with lower plasma vitamin B_{12}.[5] In a population with normal plasma values for vitamin B_{12}, the concentration of methyl malonic acid was higher in GG genotypes.[4] These studies indicate that the *TCN2* polymorphism affects availability of vitamin B_{12} in tissues for metabolic reactions.

Lack of sufficient vitamin B_{12} is associated with peripheral neuropathy, anemia, macrocytosis, depression and cognitive impairment. [6-8] It has also been reported that the elderly may present with clinical conditions and elevated concentrations of plasma biomarkers associated with vitamin B_{12} insufficiency when their plasma concentration of vitamin B_{12} is low but in the normal range.[6, 7, 9, 10] In this study we investigated the relationship between the *TCN2* polymorphism and clinical outcomes associated with vitamin B_{12} insufficiency in participants aged $\geq$ 60 y with normal plasma concentration of vitamin B_{12}. Current evidence suggests that anemia and cognitive dysfunction associated with low vitamin B_{12} are worsened by excess folate intake.[11-15] Hence we also determined the effect of folate intake on the association between the *TCN2* polymorphism and clinical outcomes.

METHODS

STUDY POPULATION

The Nutrition, Aging, and Memory in Elders Study is a cohort of community-based, home-bound elderly participants aged ≥60 y recruited from the Boston area as described in detail previously.[16] Participants were recruited from Boston's three Aging Services Access Points, which are home care agencies that provide services to the elderly to facilitate independent living. The study was conducted over 3 home visits and one hospital visit and informed consent was obtained on the first home visit. The participants were administered a neuropsychological examination, food frequency and general health status questionnaires, anthropometric measurements were taken and a fasting blood sample was collected. A subset of the participants who consented to a hospital visit were then evaluated by physicians at Tufts Medical Center. Of 989 participants from home visits who were genotyped, participants were excluded for this study if data were missing for any of the covariates (n=196), they had a stroke (n=152) which may obscure peripheral neuropathy diagnosis, had plasma vitamin B_{12} concentration below 200 pg/mL (n=8) indicative of deficiency, or above 950 pg/mL (n=59), which may be indicative of malignancies or abnormalities.[17-19] 574 participants from home visits met the criteria for the current study. This study was approved by the Institutional Review Board at Tufts University and Tufts Medical Center.

BLOOD ANALYSES

Concentrations of vitamin B_{12} and folate in plasma were measured using Immulite 1000 assay (Siemens Healthcare Diagnostics, NJ), total plasma homocysteine was determined by a high performance liquid chromatography,[20] and serum creatinine by a modified Jaffe reaction.[21] Plasma concentration of pyridoxal 5-phosphate, the functional form of vitamin B_6 was determined by a tyrosine decarboxylase apoenzyme method.[22] Hemoglobin and mean corpuscular volume were measured using HORIBA ABX Pentra 60 C+ (ABX Diagnostics, CA).

Anemia was defined as per World Health Organization as hemoglobin concentration below 13g/dL for men and 12g/dL for women and macrocytosis was defined as mean corpuscular volume >99 fL.

DNA ISOLATION AND GENOTYPING

DNA was isolated using QIAmp DNA Blood Mini kit (Qiagen, CA). *TCN2* 776C>G polymorphism was determined using a Taqman allelic discrimination assay with primers and probes specific for the polymorphism and Taqman Universal Master Mix from Applied Biosystems (CA). No-template controls and duplicate DNA samples were included in the assays for quality control. *ApoE* genotype (E2, E3 and E4) was determined as described previously.[23]

NEUROPSYCHOLOGICAL EXAMINATION

Tests for cognition were administered by a research team trained by a single neuropsychologist. Participants were excluded from the study if they scored 10 or less out of a possible score of 30 on the Mini Mental State Exam[24] (MMSE, higher scores indicate better cognition), which tests global cognition. A battery of standardized and normed tests were administered to assess cognition, and factor scores representing cognitive domains such as memory, executive function, and attention were estimated following a principal component analysis.[16] Depression was assessed using the Center for Epidemiological Studies Depression (CES-D) scale (range 0-60, with higher scores indicating greater depression).

STATISTICAL ANALYSIS

Data on plasma metabolites, hemoglobin, depression score and cognitive function were available for 574 participants. Analysis of variance was used to describe the participant characteristics by genotype categories for continuous outcomes, and Pearson's chi-square test for categorical variables. Association of *TCN2* 776C>G polymorphism with plasma concentrations of vitamin B_{12} and total homocysteine, CES-D score, and factor scores for memory, executive function, and

attention was determined using analysis of covariance. The tobit regression analysis was used to analyze association of the *TCN2* polymorphism with MMSE score. Logistic regression was used to determine the odds ratio (OR) with 95% confidence interval of having anemia, macrocytosis and peripheral neuropathy in the GG and CG genotypes compared to the reference genotype CC. To determine if folate intake modified the clinical outcomes associated with *TCN2* polymorphism we stratified the cohort based on folate intake exceeding twice the recommended dietary allowance (>800 μg vs ≤800 μg/day of dietary folate equivalents) prior to calculating the association or odds ratio as described above. For all analyses, adjustments were made for the following covariates: age, sex, body mass index, race/ethnicity, education, serum creatinine concentration, smoking status, alcohol consumption, diabetes, hypertension and vitamin B_{12} intake. We adjusted for vitamin B_{12} intake in the analyses to distinguish the effect of the *TCN2* polymorphism on the outcomes from that of vitamin B_{12} intake. Additional covariates were used where appropriate as follows: plasma concentration of folate and vitamin B_6 for plasma total homocysteine concentration, depression and cognitive function, *ApoE* genotype for depression and cognitive function and iron intake for anemia. There were 21 participants whose vitamin B_{12} intake was less than the recommended 2.4 μg/d. All the analyses were repeated after excluding these participants. All analyses were performed on SAS 9.3 (SAS Institute Inc, NC) and a two-sided $P < 0.05$ was considered significant. The data analyses were conducted by H. Sawaengsri.

RESULTS

The homozygosity for the variant allele G of the *TCN2* 776C>G polymorphism was 17.2% in this cohort, with a higher prevalence among Non-Hispanic Whites (21.5%) than Non-Hispanic African Americans (7.4%) (Table 1). The sample sizes for other races/ethnicities were too small to calculate the frequency of the variant allele. There was no difference between the genotypes in any of the other covariates used for analyses. Individuals with the GG genotype had significantly lower plasma vitamin B_{12} compared to individuals with the CC genotype, even after adjusting for

vitamin B_{12} intake (P=0.01, Table 2). Concentration of plasma total homocysteine was similar for all the genotypes (Table 2).

The *TCN2* polymorphism was not associated with clinical outcomes associated with low vitamin B_{12} such as anemia, CES-D score, factor scores of memory, executive function, and attention (Table 3 and 4). There were only 10 participants with macrocytosis in our cohort and hence a genotype effect could not be determined. MMSE scores were slightly lower in GG genotypes (n=99, 23.9±6.60) when compared to CG genotypes (n=224, 24.95±9.37, P=0.02) but not CC genotypes (n=251, 24.35±9.75, P=0.48). (Table 4).

Since high folate intake can worsen conditions associated with vitamin B_{12} insufficiency[11] we determined if the association between the *TCN2* polymorphism and the clinical outcomes was modified by folate intake. The relationship between the *TCN2* polymorphism and anemia, CES-D score, factor scores of memory, executive function, and attention were not modified by folate intake twice the recommended dietary allowance (Tables 3 and 4). Limiting analyses to only those whose dietary intake of vitamin B_{12} met the recommended dietary allowance did not change the results for any of the outcomes studied.

DISCUSSION

In this study we report the association of the 776C>G polymorphism in transcobalamin II gene *TCN2* with clinical outcomes associated with vitamin B_{12} insufficiency in older adults with normal plasma concentration of vitamin B_{12}. Individuals with GG genotype had lower plasma concentration of vitamin B_{12} when compared to those with GG genotype even though the values were in the normal range in our cohort. This could be explained by the lower transcobalamin II protein concentration as well as its impaired binding of vitamin B_{12} in the GG genotypes,[3, 25] which may result in renal excretion of vitamin B_{12}. These results are different from that of previous studies that reported no difference between genotypes or that GG genotypes have higher vitamin B_{12} concentration in plasma.[4, 5] We did not observe a relationship between

TCN2 polymorphism and plasma concentration of total homocysteine. There have been contradictory reports of higher homocysteine in individuals with GG genotypes [5, 26] as well as a lack of genotype effect.[4, 27]

In our cohort, despite normal plasma concentration of vitamin B_{12}, MMSE score which represents global cognition was significantly lower in GG genotypes when compared to CG genotypes, but the difference in the scores was small, thus may not suggest a strong clinical significance. Anemia, depression and factor scores for memory, executive function and attention were not significantly different between the *TCN2* genotypes. Our results do not support a recent report by Kurnat-Thoma *et al.* of higher MCV in the GG genotypes.[28]

High folate intake has been associated with increased prevalence of anemia and cognitive impairment in the elderly with low vitamin B_{12} status.[11, 12, 15] Recommended dietary allowance for folate is 400 µg of dietary folate equivalent, which is equal to 400 µg natural folate or 240 µg of folic acid, the synthetic form of the vitamin present in multivitamins and fortified foods. Relationship between the *TCN2* polymorphism and anemia, depression and factor scores for memory, executive function and attention tested were not influenced by folate intake. The mean dietary folate intake of our cohort was 905.5 ± 519.5 µg dietary folate equivalents, which is similar to the mean folate intake of general U. S. population older than 50, as reported for the participants of the National Health and Nutrition Examination Survey (NHANES 2003-2006),[29] indicating that excess folate intake is widely prevalent among the older adults.

A limitation of our study is the cross-sectional design of the cohort, which does not allow us to determine a cause-effect relationship. Diabetes increases the risk for cognitive impairment, but we didn't make a distinction between diabetic and non-diabetics in our analysis due to the small sample size.[30] The prevalence of diabetes was similar among all the genotypes (Table 1), hence the association of the *TCN2* polymorphism and peripheral neuropathy was not significantly affected by presence of diabetes.

In conclusion, our study shows that the *TCN2* 776C>G polymorphism is not associated with increased risk for anemia, depression and factor scores for memory, impaired executive function and attention in older adults with adequate vitamin B_{12} status.

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Table 1. Characteristics of NAME cohort by *TCN2* 776C>G Genotype

Participant Characteristics	**CC (*n*=251)**	**CG (*n*=224)**	**GG (*n*=99)**	**Overall (*n*=574)**	P Value
Age – yr	75.4±8.5	75.4±8.6	74.7±8.3	75.3±8.5	0.81
Female Sex – no. (%)	193 (76.9)	171 (76.3)	78 (78.8)	442 (77)	0.89
BMI (kg/m^2)	32.3±9.3	31.9±8.2	31.6±8.6	32.0±8.8	0.77
Education, – no. (%)					0.12
K-8^{th} Grade	32 (12.8)	27 (12.1)	8 (8.1)	67 (11.7)	
9-11^{th} Grade	60 (23.9)	33 (14.7)	23 (23.2)	116 (20.2)	
12^{th} Grade/High School Graduate	90 (35.9)	77 (34.4)	30 (30.3)	197 (34.3)	
Any Undergraduate Education	59 (23.5)	75 (33.5)	32 (32.3)	166 (28.9)	
Any Graduate Education	10 (4.0)	12 (5.4)	6 (6.1)	28 (4.9)	
Race/Ethnicity – no. (%)					<0.001
American Indian /Alaskan Native	6 (2.4)	0 (0)	0 (0)	6 (1.1)	
Asian	1 (0.4)	0 (0)	2 (2.0)	3 (0.5)	
Non-Hispanic Black	105 (41.8)	57 (24.5)	13 (13.1)	175 (30.5)	
Hispanic	0 (0)	3 (1.3)	1 (1.0)	4 (0.7)	
Non-Hispanic White	139 (55.4)	164 (73.2)	83 (83.8)	386 (67.3)	
Current Smoker – no. (%)	42 (16.7)	43 (19.2)	14 (14.1)	99 (17.3)	0.52
Dietary Consumption					

Alcohol (g/day)	1.6±6.6	2.5±9.5	2.0±9.3	2.0±8.3	0.54
Vitamin B-12 (ug/day)[2]	10.3±2.5	11.0±2.4	11.6±2.6	10.8±2.5	0.49
Folate (µg DFE/day)	867.0±519.5	927.8±536.7	952.7±476.2	905.5±519.5	0.27
Iron (mg/day)[2]	15.7±1.7	16.4±1.8	16.4±1.8	16.1±1.8	0.64
Plasma Metabolites					
Folate (ng/mL)[2]	12.1±1.8	12.8±1.7	13.8±1.7	12.7±1.7	0.13
Vitamin B_6 (ng/mL)[2]	7.4±0.4	7.7±0.4	9.0±0.4	7.8±0.4	0.13
Creatinine (mg/dL)	1.0±0.8	1.0±0.9	0.9±0.3	0.99±0.8	0.52
Hemoglobin (g/dL)	13.1±1.5	13.1±1.5	13.5±1.4	13.2±1.5	0.08
Diabetes – no. (%)[3]	71 (28.3)	77 (34.4)	35 (35.4)	183 (31.9)	0.12
Hypertension – no. (%)[3]	214 (85.3)	184 (82.1)	83 (83.8)	481 (83.8)	0.80

[1]Plus-minus values are means ±SD. P values are for the overall comparisons. Between-group differences were analyzed with the use of Pearson's chi-square test for categorical variables and one-way analysis of variance for continuous variables. The body-mass index is the weight in kilograms divided by the square of the height in meters. To convert the values for creatinine from mg/dL to micromoles per liter, multiply by 88.4. To convert the values for folate from nanograms per millileter to nanomoles per liter, multiply by 2.266. To convert values for vitamin B_6 from nanograms per millileter to nanomoles per liter, multiply by 5.982. To convert the values for vitamin B_{12} from picograms per milliliter to picomoles per liter, multiply by 0.7378.

[2] Geometric Means.

[3] Analysis of covariance adjusted for sex, age, and ethnicity/race.

[*]P=0.01 compared to the CC genotype.

Table 2. Concentrations of Plasma Metabolites by *TCN2* 776C>G Genotype					
Participant Characteristics	**CC (*n*=251)**	**CG (*n*=224)**	**GG (*n*=99)**	**Overall (*n*=574)**	P Value
Vitamin B_{12} (pg/mL)[1]	489.5±32.4	458.5±33.0	427.3±34.9*	498.3±7.4	0.01
Homocysteine (μmol/L)[2]	11.1±1.1	11.3±1.1	11.7±1.1	11.1±1.0	0.34

[1] Analysis of covariance adjusted for sex, age, BMI, education, alcohol intake, plasma creatinine concentration, race/ethnicity, smoking status, and vitamin B_{12} intake.

[2]Analysis of covariance adjusted for sex, age, BMI, education, alcohol intake, plasma creatinine concentration, race/ethnicity, smoking status, vitamin B_{12} intake, plasma folate and plasma B_6.

*P=0.01 compared to the CC genotyp

Table 3. Association between *TCN2* 776C>G Polymorphism and Anemia

	CC Genotype		CG Genotype		GG Genotype (variant)	
	Cases/ Total	Adjusted Odds Ratio	Cases/ Total	Adjusted Odds Ratio (95% CI)	Cases/ Total	Adjusted Odds Ratio (95% CI)
All participants[1]	64/251	1	61/224	1.17 (0.74 – 1.86)	17/99	0.70 (0.36 – 1.34)
≤800 µg DFE folate [2]	34/138	1	26/108	0.99 (0.50 – 1.95)	7/42	0.73 (0.27 – 2.00)
>800 µg DFE folate[2]	30/113	1	35/116	1.22 (0.62 – 2.40)	10/57	0.68 (0.27 – 1.73)

[1]Logistic regression adjusted for sex, age, BMI, education, alcohol intake, plasma creatinine concentration, plasma folate concentration, plasma vitamin B_6 concentration, race/ethnicity, smoking status, vitamin B_{12} intake, iron intake, diagnosis of diabetes, and diagnosis of hypertension.

Anemia is established by the World Health Organization as hemoglobin concentration of <12g/dL in women, or hemoglobin concentration of <13g/dL in men.

[2] Logistic regression adjusted for the covariates mentioned above except plasma folate concentration.

Table 4. Association of *TCN2* 776C>G polymorphism with Cognitive Function and Depression

Outcome	N (CC, CG, GG)	CC Genotype	CG Genotype	GG Genotype	P value
MMSE Score[1,2]	574 (251, 224, 99)	24.35±9.75	24.95±9.37	23.90±6.60*	0.01
≤800 µg DFE folate[3]	288 (138, 108, 42)	23.72±7.91	24.29±7.23	23.23±5.23	0.17
>800 µg DFE folate[3]	286 (113, 116, 57)	25.54±10.47	26.21±10.96	25.13±8.05	0.07
Memory (Factor Score)[1,5]	523 (231, 203, 89)	-0.21±2.84	-0.22±2.70	-0.34±1.91	0.58
≤800 µg DFE folate[3]	263 (128, 98, 37)	-0.22±2.31	-0.10±2.11	-0.29±1.50	0.50
>800 µg DFE folate[3]	260 (103, 105, 52)	0.29±3.21	0.23±3.33	0.12±2.47	0.62
Executive Function (Factor Score)[1,5]	523 (231, 203, 89)	-0.11±2.68	0.07±2.55	-0.04±1.80	0.12
≤800 µg DFE folate[3]	263 (128, 98, 37)	-0.31±2.10	-0.17±1.91	-0.06±1.36	0.23
>800 µg DFE folate[3]	260 (103, 105, 52)	0.13±3.15	0.39±3.28	0.07±2.43	0.08
Attention (Factor Score)[1,5]	523 (231, 203, 89)	-0.22±2.97	-0.16±2.82	-0.22±1.99	0.81
≤800 µg DFE folate[3]	263 (128, 98, 37)	-0.26±2.46	-0.32±2.25	-0.41±1.60	0.75
>800 µg DFE folate[3]	260 (103, 105, 52)	-0.20±3.33	-0.03±3.46	-0.02±2.56	0.43
CES-D Score for Depression[1,4]	539 (226, 195, 89)	11.80±18.52	11.38±17.25	11.17±11.72	0.89
≤800 µg DFE folate[3]	272 (126, 99, 38)	12.75±13.79	14.59±12.32	13.59±7.90	0.61
>800 µg DFE folate[3]	267 (100, 96, 51)	10.73±14.01	8.66±13.86	9.34±10.19	0.31

[1]Plus-minus values are mean± SD. P values are for the overall comparisons. Analysis of covariance adjusted for sex, age, BMI, education, alcohol intake, race/ethnicity, smoking status, plasma creatinine concentration, plasma folate concentration, plasma vitamin B_6 concentration, vitamin B_{12} intake, *ApoE*4 genotype, diagnosis of diabetes, and diagnosis of hypertension.

[2] Tobit regression analyses adjusted for the above covariates.

[3] Analysis of covariance adjusted for the covariates mentioned above except plasma folate concentration.

[4]Geometric mean.

[5]Factor scores for cognitive outcomes were normalized with a mean of zero and a standard deviation of 1.

[*]P=0.02 when compared with CG genotype.