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Micronutrient Status and Telomere Length in Adult Men and Women

A Dissertation Presented

by

CHRISTY S. MAXWELL

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2018

Public Health
Nutrition

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DEDICATION

This is dedicated to my ever-patient, fun-loving, amazing family - Barry, Logan, Jared, and Salle. Barry, you have been by my side for over 20 years, encouraging me to stick with it, even when life's challenges were almost too much to bear. You are my main support and my best friend forever. *As You Wish*. To my kiddos - often you found yourselves waiting patiently for mom to "finish her work" before taking you out to play. I appreciate you for all those moments and thank you from the bottom of my heart.

We finally made it!

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ABSTRACT

MICRONUTRIENT STATUS AND TELOMERE LENGTH IN ADULT MEN AND WOMEN

SEPTEMBER 2018

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Background: Telomeres are the noncoding nucleotide sequences at the end of eukaryotic chromosomes, serving to protect DNA during mitotic division. Given their guanine-rich structure, telomeres are highly susceptible to oxidative damage often mediated by inflammation. Recently, telomere attrition and dysfunction have been associated with age-related diseases, including cardiovascular disease, type 2 diabetes, Alzheimer's disease, and certain cancers. Several modifiable risk factors have also been associated with shortened telomere length, including physical inactivity, obesity, stress, smoking, and poor diet.

Objective: We examined the relations between vitamin B₁₂ status, folate status, and iron status, relative to telomere length in eligible participants in the 1999-2002 US National Health and Nutrition Examination Survey.

Design: Eligible participants had no evidence of kidney or liver disease, or pregnancy, and had complete data on outcome, exposure and covariates (n = 5,941). Low B₁₂ status was defined as serum B₁₂ ≤148 pmol/L or serum methylmalonic acid concentration ≥210 nmol/L. Folate status was defined as serum folate ≤13.4 nmol/L (suboptimal), and ≥45.3 nmol/L (elevated). Iron (Fe) status was defined as meeting ≥2 of the following criteria: serum ferritin ≤15 µg/L, erythrocyte protoporphyrin >80 µg/dL, transferrin saturation (TS) <20%, hemoglobin <12 (women) or <13 (men). Elevated Fe was defined as TS >50%, serum Fe ≥155 µg/L (women) or ≥160 µg/L (men), or serum ferritin ≥150 µg/L (women) or ≥200 µg/L (men) with no signs of inflammation.

Results: After controlling for covariates including, age, race/ethnicity, sex, BMI, education level, smoking, alcohol intake, and blood glucose level, low B₁₂ status in the presence of elevated folate status was significantly associated with a shorter mean telomere length in middle-aged adults. Elevated iron status was associated with a shorter mean telomere length in adult men and women.

Conclusion: In middle-aged adults, low vitamin B₁₂ in the presence of elevated folate status was associated with significant telomeric attrition equivalent to 27 years of additional biological aging. The shorter mean telomere length associated with an elevated iron status was equivalent to 2.6 years of additional biological aging in adult men and women. Thus, poor micronutrient status is associated with shorter mean telomere lengths in adult men and women.

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CHAPTER 1

REVIEW OF TELOMERES

A. Background

Telomeres are repetitive, non-coding nucleotide sequences at the end of all vertebrate chromosomes (TTAGGG in humans). They function as protective caps and play an indispensable role in chromosome replication and stability (McClintock, 1941). The guanine-rich 3' projection is thought to be of particular importance; acting as a buffer to protect gene coding sequences enabling gene continuity through DNA replication. The theory of a "buffering" segment of DNA was first reported by Olovnikov in 1973, where he properly ventured that with each round of cell division, a portion of this segment (telomere) would be lost, without interruption to DNA function. Furthermore, he stated that the length of each telomere would be important in determining the cell's ability to proliferate (Montpetit et al., 2014).

Telomeres have been accepted as essential components in cellular biology with the 2009 Nobel Prize in Physiology or Medicine going to Elizabeth Blackburn, Carol Greider, and Jack Szostak for their discovery of how chromosomes are protected by telomeres and the enzyme telomerase. Due to its linear formation, eukaryotic chromosomal DNA requires both the established DNA polymerase as well as the RNA enzyme telomerase to complete the replication process (Blackburn, 1991).

Oxidative stress mediated by reactive oxygen species (ROS) is responsible for single strand breaks in DNA where the telomeric GGG (guanine) end is highly susceptible to damage (Matthews et al., 2006; Von Zglinicki, 2002). This susceptibility, coupled with the knowledge that telomeric DNA has a reduced ability for repair, is

suggestive of accelerated loss and cell death under such ROS stressors. With each round of mitosis, telomeric DNA naturally shortens, resulting in cell senescence once a critical degree of telomere attrition has been reached (Blackburn, 1991). In cell culture, acute DNA damage induces premature “replicative senescence” mediated by accelerated attrition of telomeres (Matthews et al., 2006; Von Zglinicki, 2002).

Telomere attrition is now a widely accepted cellular event associated with natural aging. Moreover, telomere attrition and dysfunction have been shown to be causal factors in many age-related diseases. Thus, telomere length assessment embraces potential as a biomarker of risk assessment for numerous diverse health outcomes. There are many methods used in determining telomere length, all with advantages and disadvantages depending upon the specific study parameters.

1. Telomere Assessment

Several assessment methods may be utilized in determining telomere length. The following briefly describes the primary methods, along with their strengths and weaknesses.

a. Terminal Restriction Fragmentation

Southern Blot analysis of terminal restriction fragments (TRF) is the original method developed for determining telomere length, and thus is often referred to as the “gold standard” method. Here, genomic DNA is enzymatically digested in a manner that preserves the telomeric DNA. Intact telomeres from all chromosomes are resolved using gel electrophoresis, with fragments identified via Southern Blot analysis. Varying lengths will present as a smear, with size being compared to a DNA standard (Montpetit et al., 2014).

Strengths of the TRF method include the ability to compare the results to other investigator's data, as well as, providing a kilobase size estimate for the telomere length. Limitations include the possible overestimation in the true telomere length due to non-specificity of the enzymatic process. The DNA must be of high-quality and not degraded, thus collection and storage techniques must be carefully monitored. Results can vary between labs depending upon the restriction enzymes used. In addition, TRF requires large amounts of DNA (mg) per sample, which may prohibit its use in large-scaled studies (Montpetit et al., 2014).

b. Polymerase Chain Reaction-Based Techniques

The initial qPCR technique was developed by Cawthon et al. in 2002 and is used most frequently by researchers. Telomere length was quantified by comparing the amount of telomere amplification product (T) to a single-copy gene (S), both performed in separate tubes (Cawthon, 2002). The T/S ratio was calculated to yield a value that correlates with the average telomere length. Favorably, this technique requires minimal amounts of DNA (ng), is performed for a reasonable cost, and is amenable to high-output testing, making it well suited for large epidemiological studies. A shortcoming however, involves the possibility of intra-operator pipetting errors; thus, variations in the amount of DNA present in the "T" and "S" tubes could compromise the precision of the assay. In addition, the qPCR method results in variations, which limits the ability to allow for comparisons between studies (Montpetit et al., 2014).

c. Single Telomere Length Analysis (STELA)

The STELA method is a qPCR-based method that was developed to detect the length of specific, individual telomeres. While the first two methods discussed provide an

average telomere length, this method provides the means for recognizing the presence of short telomeres on a single chromosome. This method is useful in studies investigating the presence of critically short telomeres of specific cells in low concentrations.

Limitations include the inability to identify long telomere lengths, as well as its labor-intensive, technically challenging protocol (Montpetit et al., 2014).

d. Quantitative Fluorescence in situ Hybridization (Q-FISH) Methods

Q-FISH methods involves assessing metaphase chromosomes (or interphase nuclei) following a fluorescence labeling. In contrast to the TRF and qPCR methods, Q-FISH utilizes cells as the substrate, rather than DNA. In general, Q-FISH assays have the advantage of estimating sizes for each of the individual 92 telomeres in humans (and not limited to an average or short lengths). These assays have been essential in providing information about variation in length of telomeres between different chromosomes and is particularly valuable in measuring telomere length in rare cells. However, the metaphase Q-FISH approach is labor intensive, expensive, and difficult to perform, making it impractical for large-scale epidemiology studies. An adapted version of this Q-FISH method is called flow-FISH, which has the capability of sorting cells into subpopulations based on size, granulation, and/or antibody labeling. Flow-FISH was the first assay to be used clinically in diagnosing individuals with dyskeratosis congenital – a condition associated with shortened telomere lengths (Montpetit et al., 2014).

Further Q-FISH approaches, as well as other methods including the hybridization protection assay (HPA) and single-strand 3' overhang measurement methods are also available. As with the methods described above, each have strengths and weaknesses

associated with measuring telomere length, but are utilized much less frequently in epidemiological studies (Montpetit et al., 2014).

2. Non-Modifiable Determinants of Telomere Length

a. Sex and Telomere Length

Aging is a non-modifiable factor that inevitably results in telomeric attrition. Sex and race are two other factors that are genetically predetermined and may influence telomere length. Gardner et al. (2014) conducted a systematic review and meta-analysis on sex and telomere length. Some studies have found longer leukocyte telomeres in women than in men, while others have found no association, or even the reverse. Theories as to why women may have longer telomeres include the role of estrogen. Telomerase contains an estrogen-responsive element that is thought to stimulate telomerase activity (Mayer et al., 2006). Additionally, telomeres are highly sensitive to oxidative stress and women produce less reactive oxygen species (ROS) than men (Nawrot, Staessen, Gardner, & Aviv, 2004). It has also thought that the antioxidant properties of estrogen may allow women to metabolize ROS more efficiently, thus protecting the susceptible telomeric strands.

From a potential 10,149 studies, Gardner et al. included 40 datasets in the meta-analysis. Telomere length was determined by Southern Blot (17 datasets), qPCR (19 datasets) or Flow-FISH (four datasets) in the eligible studies. There was little evidence that the association between sex and telomere length varied by age group, but it did vary by measurement method. The summary estimates of effect showed longer telomeres in females than in males only in the Southern Blot method. The investigators concluded that the failure of the qPCR and Flow-FISH methods in finding longer telomere lengths in

females compared to males was due to higher experimental variability in the techniques (Gardner et al., 2014).

b. Race/Ethnicity and Telomere Length

Racial disparities have been associated with lower life expectancy, higher rate of disability, and earlier onset of chronic disease (Murray et al., 2006; Rewak et al., 2014). One theory as to these disparities is the “weathering” hypothesis that Geronimus et al. (1992) first suggested. This theory explaining such disparities, suggests that differences found in populations in the early stages of chronic disease, result from different life experiences, stress exposures, and access to coping resources associated with social identities or “assignments” such as race/ethnicity, from conception through adulthood. Nonetheless, data on race/ethnicity and telomere length have differed. Several studies have found longer telomere lengths in black compared to white participants (Adler et al., 2013; Aviv et al., 2008; Hunt et al., 2008; Needham et al., 2013). In contrast, Geronimus argued these findings were the result of highly selected samples that included few to no social covariates (Geronimus et al., 2015). In this study, Geronimus et al. investigated the relationship between race-ethnicity, poverty, and urban stressors and telomere length in a community-based study in Detroit, Michigan. In a sample of 202 black, white, and Mexican adults taken from three Detroit neighborhoods, the investigators measured telomere length and self-reported survey data including information on SES, psychosocial, neighborhood, and behavioral stressors. The investigators found that after stratifying each racial/ethnic group on a poverty-to-income ratio (PIR), black participants had shorter mean telomere length compared to nonpoor white participants. However, this finding was not significant. The authors found that all racial/ethnic groups were sensitive

to the addition of socioeconomic, psychosocial, coping, and biobehavioral variables when added to the model.

Interestingly, the authors also found that poorer whites had significantly shorter telomere lengths than non-poor whites ($p < 0.01$); whereas this relationship with poverty was not observed among the black or Mexican participants. The authors cite that whites may be more sensitive to their perceived socioeconomic failures when the expectations of white privilege and the current circumstances are not congruent (Geronimus et al., 2015). Thus, great care in study design and analyses should be taken when investigating future relationships on telomere length among different race/ethnicities.

3. Modifiable Determinants of Telomere Length

a. Overview

Telomere length is genetically determined (Vasa-Nicotera et al., 2008); however, telomere attrition has been associated with many environmental and lifestyle factors, including obesity (Revesz, Milaneschi, Verhoeven, Lin, & Penninx, 2015), low physical activity (Cherkas et al., 2008), poverty and educational status (Arlene T. Geronimus et al., 2015), stress (Epel et al., 2004), and smoking (Huzen et al., 2014). Shorter telomere length has also been associated with chronic disease states such as cardiovascular disease (Fitzpatrick et al., 2007), hypertension (Samani, Boulby, Butler, Thompson, & Goodall, 2001), type 2 diabetes (Willeit et al., 2014; Zhao et al., 2014), Alzheimer's disease (Panossian et al., 2003), and certain cancers (Willeit et al., 2010) independent of chronological age. The disputable issue of whether telomere length is causal or consequential of aging remains elusive. Nonetheless, evidence indicates that telomere length is a key trademark of aging (López-Otín, Blasco, Partridge, Serrano, & Kroemer,

2013). An observation that has paved the way for its use in epidemiological research investigating the role of telomere length in predicting disease and mortality, as well as whether it can be altered by lifestyle and dietary modifications.

b. Physical Activity

Environmental and lifestyle factors have been investigated for their association with telomere length in numerous studies. Research supports the role of physical activity (PA) and numerous health benefits. Thus, a positive association between PA and telomere length has been proposed (Cherkas et al., 2008). In an effort to critically assess the data on PA and telomere length, a systematic review and meta-analysis on the subject was conducted (Mundstock et al., 2015). The authors included 37 original studies in the systematic review (n=41,230), and 25 had sufficient data to be included in the meta-analysis, 11 of which specifically evaluated the correlation between the level of PA and telomere length. Of the 37 studies in the systematic review, 15 demonstrated a positive association between reported PA and telomere length, 14 of which were significant. The authors state that based on their results, there was not enough good-quality evidence to clearly establish an association between PA and telomere length. The meta-analysis demonstrated a difference in the standard means between the active and inactive groups of 0.91 (95% CI 0.48-1.35) in favor of the active group, however, this was not statistically significant. This systematic review and meta-analysis was limited primarily due to the moderate quality and methodological limitations of the included studies. There was high heterogeneity in the meta-analysis likely due to the differences in methodological techniques including measurement of telomere length, lack of controls,

poor information on potential confounding variables, and the instruments used in determining the level of PA (Mundstock et al., 2015).

c. Obesity

To assess the effects of obesity on telomere length, investigators screened 789 potential unduplicated articles and conducted a systematic review and meta-analysis on the data. Of these, 716 studies were excluded for various reasons including missing data and not meeting inclusion criteria. Sixty-three studies were included with data on 119,439 participants. Of the studies examined, 24 did not find significant results, while the remaining 39 found a statistically significant association between obesity and telomere length (38 of which had an inverse relationship). The meta-analysis of studies reporting an association between body mass index (BMI) and telomere length yielded a trend for a weak inverse correlation. As in the previous systematic review on physical activity, this study also reported high heterogeneity indicating the results should be considered with caution. The question on a causal relationship between obesity and telomere length remains elusive.

d. Smoking

Poorer health outcomes have long been attributed to smoking status. In the mid-1980's Nakayama et al. noted that smoking resulted in single-strand breaks in human DNA (Nakayama, Kaneko, Kodama, & Nagata, 1985). In a recent paper, investigators looked at the effects of smoking as well as other metabolic traits (waist circumference, cholesterol levels, blood glucose levels, and blood pressure) on telomere length. Active smoking increased the yearly telomere attrition rate by more than three-fold (Huzen et al.,

2014). The authors also mention that for subjects who were former smokers, their annual attrition rate was comparable to subjects who never smoked.

In addition to smoking, the authors found evidence supporting longitudinal associations between accelerated telomere loss and high blood pressure, elevated glucose levels and increased waist circumference (Huzen et al., 2014). These findings support the hypothesis that certain modifiable lifestyle factors impact the speed of biological aging and could provide a plausible mechanistic link to many age-associated diseases. Future prospective research is needed to determine if modifying telomere length could influence healthy aging and the associated disease states.

e. Indicators of Socioeconomic Status

As with other environmental factors, previous studies have shown an association between indicators of socioeconomic status (SES) and morbidity and mortality (Adler & Rehkopf, 2008). SES is often measured via numerous indicators such as education, income, social class, and occupation, depending on the available data, research question, and/or geographical location (Robertson et al., 2013). These assessments are considered broad indicators of SES and often used interchangeably. Education, however, has been hypothesized as being a better indicator of SES in adulthood, and thus a superior marker for identifying an association between SES and telomere length. Effects of SES on telomere length may take years to accumulate; therefore, education may best represent the time during early adult life through middle age more so than present day indicators (Steptoe et al., 2011).

Several studies have investigated the association of telomere length and indicators of SES. In one study, researchers examined 70 children (35 white, 35 black) with ages

ranging from 7 to 12.6 years, who were randomly selected from a larger pool of 360 children (120 white, 120 black, 120 Latino) (Needham, Fernandez, Lin, Epel, & Blackburn, 2012). SES measures included parental education level and household income. Children living in the lowest income households (<\$40,000/year) tended to have a lower T/S ratio compared to children living in the highest income families (\geq \$70,000/year) ($\beta = -0.38$; 95% CI: -0.82, 0.06; $p = 0.09$) (Needham et al., 2012).

Needham and colleagues also utilized data from the National Health and Nutrition Examination Survey (NHANES) and investigated the association between SES, health behaviors and telomere length (Needham et al., 2013). The authors used education level and household income as indicators for SES, as well as BMI, smoking, alcohol and physical activity (PA) as indicators for health behaviors. Telomere lengths of respondents who did not graduate from high school were approximately 4% shorter than those who graduated from college ($p < 0.01$) (Needham et al., 2012). This corresponded to a difference of 102 base pairs (bp), which given the model-based estimate of age-associated telomere shortening of 14.6 bp/year, was roughly equivalent to seven years of additional aging. The authors found no association between income and telomere length.

Despite the association found from this large, nationally representative sample, a systematic review and three separate meta-analyses of 29 unique studies found no evidence for an association between SES and telomere length. However, the meta-analysis on education confirmed that high (compared to low) education was weakly associated with longer telomere length (Robertson et al., 2013). Notably, there was significant heterogeneity among the studies. Although these analyses provide an indication to support a positive relationship between education and telomere length, they

fail to provide conclusive evidence of an association between all SES indicators and telomere length, warranting further investigation.

f. Stress and Mental Health

Epidemiological studies have investigated an association between stress and chronic mental states, such as depression and anxiety disorders, as well as, cardiovascular disease and type 2 diabetes. Data from the 1999-2002 cycles of NHANES yielded an association between telomere length and mental health disorders (Needham et al., 2015). After controlling for age, gender, and race/ethnicity, no associations between depression or anxiety status and telomere length were found in a sample of 1,164 participants. When stratified on gender (a known confounding variable), generalized anxiety disorder / panic disorder was significantly associated with shorter telomere length in women (β : -0.26), but not in men (β : -0.00). In addition, the researchers found that severe depressive symptomatology (identified by antidepressant use) was associated with shorter telomere length even in early adulthood. The authors state that their study was marginally underpowered (Needham et al., 2015).

Investigators of another study looked at the association of racial discrimination and mental health on telomere length in a sample of 95 African American men (Chae et al., 2016). After controlling for sociodemographic factors, greater anxiety symptoms were associated with shorter telomere length (β : -0.29, SE = 0.014, $p < 0.05$). In addition, there was a significant interaction between racial discrimination and depression predicting telomere length with reported racial discrimination being associated with shorter telomere length among those with lower levels of depressive symptoms (β : 0.011, SE = 0.005, $p < 0.02$). As hypothesized, participants reporting low levels of racial

discrimination along with lower levels of depressive symptoms, had a longer mean telomere length (Chae et al., 2016).

g. Oxidative Stress

Of the proposed mechanisms relating stress and overall health, alterations in the hypothalamic-pituitary-adrenal (HPA) axis and the immune system are of relevant interest as inflammation is a key biological mediator of morbidity and mortality. Inflammation is known to trigger T-cell proliferation, which is linked to telomeric shortening (Kiecolt-Glaser et al., 2013). Inflammation can also result in oxidative stress, defined as “a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses” that may lead to tissue injury (Betteridge, 2000). Production of ROS, such as the hydroxyl radical ($\bullet\text{OH}$) and superoxide anion ($\text{O}_2^{\bullet-}$), is an unavoidable circumstance of the body’s biochemical processes. These free radicals are unstable due to the presence of unpaired electrons and can start a chain of events ultimately leading to cell damage. Antioxidant defenses are the body’s primary protection against tissue injury. Chronic low levels of oxidative stress can cause DNA damage, resulting in single-strand breaks (Petersen, Saretzki, & Zglinicki, 1998). Telomeres are particularly susceptible to oxidative damage given their guanine-rich ends (Von Zglinicki, 2002). This author noted that the loss of protective telomeres may result in chromosome instability, fusion with neighboring chromosomal ends and double-strand breaks in DNA.

A reduction in oxidative stress leads to a decreased rate of telomeric shortening and postponement of cell senescence (Sitte, Saretzki, & Von Zglinicki, 1998). As both an indicator of oxidative stress and cell senescence, telomere length has emerged as a

potential biomarker of biological, as well as functional aging. Dysfunctional telomeres are linked to development of chronic pathologies including cardiovascular disease, type 2 diabetes, hypertension, Alzheimer's disease and certain cancers (Paul, 2011).

B. Health Outcomes and Telomere Length

1. Cardiovascular Disease

A systematic review and meta-analysis was conducted investigating telomere length and risk of cardiovascular disease (CVD) (Haycock et al., 2014). The authors focused on prospective and retrospective studies involving coronary heart disease (CHD) and/or cerebrovascular disease. In all, 24 studies (12 prospective, 12 retrospective) involving 43,725 participants (8400 with CVD) were investigated. The heterogeneity in telomere length were reported to be attributable to the quality, size, and age variability. The pooled relative risk (RR) for CHD comparing the subjects with the shortest third of telomere length to the subjects in the group with the longest third of telomere length was 1.54 (95% CI: 1.30, 1.83; 20 studies) with moderate heterogeneity ($I^2 = 64%$) (Haycock et al., 2014). Additionally, the pooled RR for cerebrovascular disease for shortest third vs longest third of telomere length using information from 10 studies (4 retrospective, 6 prospective) after adjusting for multiple factors was 1.42 (95% CI: 1.11, 1.81) with no significant heterogeneity present (Haycock et al., 2014).

Results from this meta-analysis indicate an inverse association between telomere length and risk of CHD independent of conventional vascular risk factors. The association between telomere length and cerebrovascular disease was comparable; however, it was not significant in the meta-analyses restricted to the prospective studies

or the higher quality studies. The authors indicated further well-designed, prospective studies are warranted to determine causality. They speculate that one mechanism for a causal association between shorter telomeres and increased CVD risk could be the accumulation of senescent cells, which are a prominent feature of atherosclerotic plaque. This cell accumulation promotes apoptosis, which can further exacerbate inflammation and endothelial dysfunction (Haycock et al., 2014).

2. Type 2 Diabetes Mellitus

Data from the Danish Twin Registry was used to investigate the association between development of insulin resistance and telomere length (Verhulst et al., 2016). The authors analyzed data from 338 same-sex twin pairs who participated in a 12-year longitudinal study on cardiovascular risk factors. Plasma glucose, cholesterol and triglyceride levels, and serum insulin were measured, and HOMA-IR was calculated as a measure of insulin resistance. Baseline insulin resistance was not associated with telomere length attrition at follow-up; however, shorter telomere length at baseline was associated with an increased development of insulin resistance at follow-up ($p < 0.0001$). These data support the hypothesis that those who have a shorter telomere length in young adulthood may be more susceptible to developing insulin resistance and conceivably type 2 diabetes at a younger age compared to their peers with a longer telomere length.

Similar results were found from a prospective cohort of 2,328 Native Americans, a population with a disproportionately high prevalence of diabetes (Zhao et al., 2014). At baseline, diabetic participants had a significantly shorter mean telomere length, higher body mass index (BMI), larger waist circumference (wc), higher systolic blood pressure,

higher triglycerides, and higher highly-sensitive C-reactive protein (hsCRP). After adjusting for age and other covariates, the inverse associations between telomere length and BMI ($r=-0.121$), wc ($r=-0.154$), and hsCRP ($r=-0.089$) remained significant (Zhao et al., 2014).

Among the 2,328 initially non-diabetic participants, 292 developed type 2 diabetes (T2DM) at the end of the five-year follow up (12.5%). After adjusting for covariates, those that developed T2DM had a significantly shorter mean telomere length at baseline (mean T/S ratio 0.96 vs. 1.02) compared to those who remained free of the disease ($p = 0.007$). Adjusted cox spline regression analysis, using telomere length as a continuous variable, indicated that a longer mean telomere length was significantly associated with a reduced risk of T2DM ($\beta = -2.78$; HR 0.06 [95% CI: 0.01, 0.42], $p=0.004$) (Zhao et al., 2014). The results demonstrated that those in the shortest quartile for telomere length had a nearly two-fold increased risk of developing T2DM compared to the those in the longest quartile (HR 1.83 [95% CI: 1.26-2.66], $p<0.0001$) (Zhao et al., 2014).

In a separate prospective study, 1000 participants were followed every five years for 15 years, with a 90% participation rate (Willeit et al., 2014). Leucocyte telomere length from three time points were collected and analyzed (1995, $n=684$; 2005, $n=558$; 2010, $n=479$). Of the 606 participants who were free of T2DM at the start of the study, 44 went on to develop the disease (7.3%). The authors reported an adjusted hazard ratio (HR) for T2DM of 2.00 (95% CI: 0.90, 4.49; $p = 0.091$) comparing the shortest telomere length quartile to the longest. When combining the top three quartiles and comparing them to the shortest, they reported a HR of 2.31 (95% CI: 1.21, 4.41; $p = 0.011$) (Willeit

et al., 2014). The authors also conducted a literature-based meta-analysis of three relevant prospective studies, including the previously discussed study by Zhao and colleagues. The pooled studies included 6,991 participants and 2,011 incident cases of T2DM. The pooled RR for comparison of the shortest telomere length quartile at baseline with the longest was 1.31 (95% CI: 1.07, 1.60; $p=0.01$; $I^2 = 69\%$) (Willeit et al., 2014). The authors report that shorter relative telomere length is independently associated with the increased risk of T2DM; however, a causal involvement of telomeres in the disease development remains elusive (Willeit et al., 2014).

3. Cancer

Attention was given to a 2009 publication by Hou et al. for their proposition that shortened telomere length is a risk factor for gastric cancer (Hou et al., 2009). The authors conducted a population-based case-control study in Warsaw, Poland between 1994 and 1996. Telomere length was measured using quantitative real-time polymerase chain reaction (qPCR) in 300 cases of gastric cancer and 416 age- and sex-matched controls, investigating the association between telomere length and risk of gastric cancer, as well as confounders and risk modifiers for telomere length-related risk. Data on education level, BMI, smoking status, alcohol intake, calorie intake, dietary patterns including fruit and vegetable intake, red meats, sausage, and preserved vegetables, as well as *H. pylori* infection status and familial history of cancer were included in the analyses.

As expected, when compared to the controls, cases had lower levels of education, drank more alcohol, smoked more cigarettes, consumed fewer vegetables, and had greater familial history of gastric cancer. With regards to telomere length, cases of gastric cancer

had significantly shorter telomere length compared to controls. Unconditional logistic regression analysis of telomere length in quartiles, demonstrated that the risk of gastric cancer was doubled for cases in the lowest quartile of telomere length compared to those in the highest quartile (OR=2.04; 95%CI: 1.33, 3.13) even after adjusting for many potentially confounding factors (Hou et al., 2009).

A similar study was conducted investigating telomere length and risk of gastric cancer (Liu et al., 2009). The authors reported that after adjusting for confounding factors, individuals with a shorter telomere length had a two-fold increased risk of gastric cancer (OR = 2.14, 95% CI: 1.52, 2.93) (Liu et al., 2009). The authors also observed a dose-response relationship with risk of gastric cancer within the quartiles of telomere length. With the lowest quartile being the referent group, the second, third, and fourth quartiles had a subsequent increased risk of gastric cancer with an OR of 1.45 (95% CI: 0.97, 2.23), 2.11 (95% CI: 1.36, 3.24), and 3.12 (95% CI: 2.01, 4.79), respectively (Liu et al., 2009). The results of these two studies on telomere length and gastric cancer are in agreement with other studies of telomere length and cancer, including cancers of the bladder (Broberg, Björk, Paulsson, Höglund, & Albin, 2005; McGrath, Wong, Michaud, Hunter, & De Vivo, 2007), lung (Jang et al., 2008), kidney (Pal et al., 2015), and pancreas (Skinner et al., 2012).

Among renal cell carcinoma (RCC) cases, data suggested significantly shorter telomere lengths within the RCC tissue compared to normal adjacent renal tissue from the same subject ($p = 0.000$) (Pal et al., 2015). Low grade tumors (grades I & II) had significantly longer telomere lengths than high grade tumors (grades III & IV) ($p = 0.016$), indicative of extensive cell proliferation. The authors suggest their findings

support the view that shorter telomere length is in part, responsible for early development and aggressiveness of the tumor, and that telomere length and telomerase activity can be used as predictive and diagnostic markers of cancer (Pal et al., 2015).

A U-shaped phenomenon has also been observed in studies investigating telomere length and cancer. Data from a case-control study on telomere length and gastric cancer risk in a Chinese population demonstrated an increased risk of gastric cancer associated with both shorter and longer telomere lengths (Du et al., 2015). The study population consisted of 1136 cases of gastric cancer along with 1012 age- and gender-matched cancer-free controls. The researchers separated the groups into a quintile distribution of relative telomere length (RTL) for analyses. The first quintile contained those with the longest RTL, while the second quintile was the referent group. There was a dose-response increased risk of gastric cancer observed in the third, fourth, and fifth groups with odds ratios of 1.28 (95% CI 0.93, 1.77), 1.65 (95% CI: 1.21, 2.26), and 3.81 (95% CI: 2.82, 5.13), respectively when compared to the referent group (Du et al., 2015). Interestingly, the first quintile, those with the longest RTL, had an OR of 1.78 (95% CI: 1.30, 2.44). Thus, a near two-fold increased risk of gastric cancer was found among those with the longest telomere length (Du et al., 2015).

U-shaped results have also been reported in other studies involving telomere length and cancer. Investigators observed a linear trend for a higher risk of pancreatic cancer associated with a shorter mean telomere length where the overall result was a skewed U-shaped relationship between telomere length and prevalence of pancreatic cancer (Skinner et al., 2012).

Other studies have reported finding associations of longer telomere length with cancer. Investigators conducted a case-control study in women looking at telomere length and risk of breast cancer among 265 cases and 446 controls (Svenson et al., 2008). Blood cell telomeres were longer in the cases compared to the controls. In determining associated risk of breast cancer, the groups were divided into quartiles based on relative telomere length (RTL) distribution among the controls. Breast cancer risk increased in the second, third, and fourth quartiles compared to the referent group ($P_{\text{trend}} < 0.001$), with an OR of 5.17 (95% CI: 3.09, 8.64) in the quartile with the longest telomeres (Svenson et al., 2008). The researchers also examined RTL as a predictor of survival. The cases were dichotomized into two groups based on the median RTL value of 0.73. Patients under 50 years of age with shorter telomeres had a significantly better outcome than patients with telomeres longer than the median value ($p = 0.024$). For patients 50 years and older, a borderline value was observed ($p = 0.095$). Overall, the investigators determined that RTL was found to represent a strong independent prognostic indicator in patients with advanced breast cancer.

4. Alzheimer's Disease and Cognitive Decline

Cognitive decline, including dementia and Alzheimer's disease, is highly associated with the aging process and is significantly modified by genetic and environmental factors. A study in a cohort of elderly Chinese men looked at telomere length and cognitive function (Ma et al., 2013). The 976 participants were originally recruited for a health survey in Hong Kong between 2001 and 2003 where they were

screened for cognitive function via the Mini-Mental State Examination (MMSE), as well as the Community Screening Interview for Dementia.

Participants were divided into tertiles based on mean telomere lengths of short (7.20 kb, n = 335), medium (8.85 kb, n = 369) and long (11.00 kb, n = 251). No association was found between telomere length and MMSE score; however, subjects in the medium and long telomere length groups performed significantly better in the episodic memory challenge ($r=0.086$, $p=0.007$), as well as the executive function measure of verbal fluency ($r=-0.053$, $p=0.048$) after adjusting for age and education level (Ma et al., 2013).

When subjects were divided by MMSE score using the accepted cutoff for cognitive impairment (>24 vs ≤ 24), those below the cutoff had a mean telomere length 251 base pairs (bp) shorter than those with an MMSE score above the cutoff ($p = 0.04$). Additionally, when results from the three exams were combined, participants with all three test scores above the cutoff had a significantly longer mean telomere length (9.03 kb, n=244) compared to those with no scores above the cutoff (8.67 kb, n=52) (Ma et al., 2013). Notably, this study failed to control for potential factors associated with the outcome including BMI, co-morbidities, and smoking status.

In a longitudinal study from the Lothian Birth Cohorts 1936 and 1921, data were used to investigate telomere length and cognitive decline later in life (Harris et al., 2016). Blood samples, as well as physical and cognitive exams were collected and analyzed at multiple timepoints throughout the study. Quantitative PCR was used to analyze telomere length. Linear mixed models were used to investigate change in telomere length, cognitive function and physical ability over time. In both cohorts, there was an overall

decrease in telomere length with age. Interestingly in both cohorts, telomere length was longer in males than in females (LBC1936 by 177.9 bp; LBC1921 by 256.9 bp). This finding is contrary to most data available on sex and telomere length (Gardner et al., 2014). There was no evidence to indicate that baseline telomere length was associated with the course of physical and/or cognitive decline. Thus, the rate of telomere length decline did not correlate with the rate of decline in physical and/or cognitive function.

The longitudinal design adds strength to the results. The investigators were able to utilize multiple time points throughout the duration to measure telomere length in aging adults. Nevertheless, this study experienced loss to follow up due to natural attrition in these aged cohorts (retention of 64% and 11%, respectively). The authors neglected to mention the possibility of survival bias, which may have impacted their findings and nullified their results.

C. Micronutrients and Telomere Length

1. Overview of Dietary Intake and Telomere Length

It is well known that numerous dietary factors influence the risk of chronic disease, but the mechanisms of action by which micronutrients influence chronic disease outcome are complex and uncertain in most cases. Moreover, little is known about the association of telomere length and micronutrient status in adults. In a study of 56 middle-aged Italian subjects, Marcon and colleagues found a significant correlation between mean telomere length and dietary intake of micronutrients, based on a food frequency questionnaire (FFQ) developed for Italian dietary habits (Marcon et al., 2012). The authors reported that vegetable intake was significantly and positively associated with

mean telomere length after adjusting for age ($p=0.013$), with strong associations also found among the antioxidants beta-carotene ($p=0.001$), vitamin A ($p=0.001$), vitamin C ($p=0.016$), vitamin E ($p=0.014$), as well as folic acid intakes ($p=0.019$) (Marcon et al., 2012). In this study, the term folic acid was used to represent folate for which *intake* was determined based on consumption of dark leafy greens, categorized as “spinaches” by the researchers. Currently, cereal grains are not fortified with folic acid in Italy, and only a limited number of commodity items available, such as corn flakes, have been fortified with folic acid.

Interestingly, when the women were dichotomized on age (above and below the mean age of 56 years), beta-carotene appeared to be protective against telomere attrition only in the younger women ($p=0.009$) (Marcon et al., 2012). This observation supports the role of dietary factors, specifically antioxidants, in diminishing oxidative damage to telomeres in younger individuals. Notably, this study did not investigate biomarkers for any of the micronutrients. Nonetheless, the authors referenced a study which found a significant correlation between the dietary FFQ used and blood micronutrient levels. However, the use of “spinaches” as a dietary marker of folate intake alone is limiting. Dark leafy greens are rich in numerous micronutrients and phytochemicals. The possibility remains that the correlation between “folate” intake and telomere length proposed by the authors may be due to other nutritive components in the dark leafy greens, and not limited to folate itself.

In another study, Xu et al. investigated multivitamin use and telomere length in 586 women 35 to 74 years of age (2009). The authors reported that in general, multivitamin use was associated with longer telomere length, and compared to non-users,

daily users had on average 5.1% longer telomeres ($P_{\text{trend}} = 0.002$). Statistically, this difference corresponded to approximately 9.8 years of age-related telomere loss. Once-a-day multivitamins and the antioxidant combination supplements were also significantly associated with telomere length ($P_{\text{trend}} = 0.01$, $P_{\text{trend}} = 0.009$, respectively); however, the authors found no association between the Stress-tabs or B-complex use category and telomere length ($P_{\text{trend}}=0.5$). When individual micronutrient supplements were investigated, only vitamin B₁₂ and iron use demonstrated significant findings. Vitamin B₁₂ users (n = 52) had significantly longer telomere lengths ($p = 0.03$) compared to non-users of vitamin B₁₂ supplements (n = 518). In addition, iron users (n=41) had significantly shorter telomere lengths ($p = 0.007$) compared to non-users of iron supplements (n = 527). Although most micronutrient intakes from food in the Xu study were positively associated with telomere length, most became nonsignificant when adjusted for multivitamin use. Nonetheless, among the women who did not use multivitamins (n = 203), higher dietary intakes of beta-carotene ($p = 0.045$), folate ($p = 0.02$), magnesium ($p = 0.04$), vitamin C ($p = 0.002$), vitamin A ($p = 0.008$), and vitamin E ($p = 0.03$) were significantly associated with longer telomere length (Xu et al., 2009).

Similarly, Garcia-Calzon and associates reported diets higher in antioxidants were significantly associated with longer telomere length (García-Calzón, Molerés, et al., 2015). The authors demonstrated that dietary total antioxidant capacity (TAC) was positively correlated with longer telomere length in Spanish children ages 6 to 18 years ($p = 0.007$) after adjusting for covariates. In addition, higher intakes of legumes and PUFAs were positively associated with longer telomere length ($p = 0.032$ and $p = 0.019$, respectively), while diets higher in cereals and white bread were significantly associated

with shorter telomere length ($p = 0.002$ and $p = 0.002$, respectively), even after adjusting for potential confounders (García-Calzón et al., 2015).

2. Iron Status and Telomere Length

The average human body contains approximately two to four grams of iron. Although iron is found in numerous food sources, the typical American diet consists of only five to seven mg/1000 kcal in both a heme and nonheme form. Heme iron is that which is bound to the porphyrin ring structure and derived from hemoglobin and myoglobin sources found in animal products. Nonheme iron is sourced primarily from plant foods including leafy greens, nuts, legumes, grains, fruits and vegetables (Gropper, Smith, & Groff, 2009a).

Digestion and absorption of the two forms differ; thus, dietary intakes and restrictions can substantially influence iron stores. Heme iron is first hydrolyzed in the stomach and small intestine to release the heme-porphyrin ring (also known as metalloporphyrin) from the globin complex. Heme is readily absorbed across the brush border of the enterocyte by a heme carrier protein (hcp1), primarily in the duodenal region of the small intestine.

Nonheme iron must be enzymatically released from food to be absorbed. Most nonheme iron from plant sources is in the ferric ($+3$) form, which is soluble under acidic conditions, such as in stomach acid. However, once chyme passes into the small intestine, the alkaline pancreatic secretions may cause ferric iron to form ferric hydroxide ($\text{Fe}(\text{OH})_3$), an insoluble, less bioavailable iron complex. Ferrireductases, such as Dcytb, on the brush border in the duodenum function to reduce ferric iron to the more soluble ferrous state. Vitamin C appears necessary for the reductase activity. Ferrous iron from

nonheme sources crosses the brush border via the divalent mineral transporter 1 (DMT1) in the duodenal portion of the small intestine. DMT1 also transport other minerals such as zinc, manganese, copper, nickel, and lead.

Within the enterocyte, ferrous iron (Fe^{+2}) binds to compounds (chelators and ligands) which determine iron's fate depending on the binding nature of the chelator/ligand. Iron can be used by the mucosal wall, sloughed off within the cell and excreted into the feces, or transported out and used by other body tissues. Absorption enhancers for nonheme iron include sugars, such as fructose, acids, such as citric acid and ascorbic acid, as well as products of meat digestion. Dietary factors inhibiting nonheme iron absorption include polyphenols (tannin derivatives), oxalates from foods such as spinach, berries and chocolate, phytates from corn, whole grains and legumes, as well as other minerals including calcium, zinc and copper (Gropper et al., 2009a).

Nearly 65% of iron in the body is associated with hemoglobin; however, the importance of iron extends well beyond its oxygen carrying ability. The remaining iron is stored in hepatocytes as ferritin and hemosiderin (as Fe^{+3}), or in the spleen, bone marrow, and/or muscle tissue, as well as being a part of enzymes. Iron is involved in numerous metabolic processes as a part of heme in a cluster with sulfur, or by itself. It functions as a component of proteins involved in DNA synthesis, such as ribonucleotide reductase, and part of the catalytic site of cytochromes and peroxidases. Iron also acts as a cofactor for dozens of enzymes, including the reactive oxygen species (ROS) quenching enzymes catalase and myeloperoxidase (Gropper et al., 2009a).

Reactive oxygen species such as the superoxide radical (O_2^\bullet), hydroxyl radical ($^\bullet\text{OH}$), and hydrogen peroxide (H_2O_2) are generated in the body and are typically

quenched by antioxidants and/or enzymes. However, a disruption in the equilibrium and subsequent ROS overload, can result in oxidative damage. Ferric iron (Fe^{+2}) is released in high concentrations from lysosomal apoptosis resulting from the cell damage. This excessive free ferrous iron can be a catalyst for further reactions (i.e., Fenton reaction) from which ROS are formed; thus perpetuating the cycle and leading to oxidative stress (Kepinska, Szyller, & Milnerowicz, 2015).

Antioxidants appear to have a protective effect and have been associated with longer telomere length (García-Calzón et al., 2015; Xu et al., 2009). Iron has the unique properties of being involved in both anti-oxidative and pro-oxidative reactions (Pra, Franke, Henriques, & Fenech, 2012). It is also a key trace element in nucleic acid metabolism, including nucleotide synthesis, as well as DNA synthesis and repair (Pra et al., 2012). Iron is a co-factor in DNA polymerases, DNA helicases, and the DNA primase regulator subunit PRIM2 (Zhang, 2014). Iron deficiency, as well as iron overload, is associated with genomic instability. Iron deficiency anemia increases nuclear DNA damage in adults (Zhang, 2014). Thus, both conditions have the potential to have a detrimental effect on telomere length. To our knowledge, no study has investigated the impact of iron deficiency on telomere length.

a. Iron Status and Telomere Length

The findings on iron supplementation by Xu et al. (Xu et al., 2009) were in agreement with data reported by Mainous and colleagues in which elevated serum biochemical tests of iron status were significantly associated with shorter telomere lengths ($p < 0.0001$), likely due to the highly oxidative nature of iron (Mainous III et al., 2013). From the available data on 511 men and women, the authors investigated both

phenotype (serum ferritin >1000 ng/mL) and *HFE* genotype (gene for hemochromatosis) markers of iron status. The researchers found that an elevated iron phenotype, but not hemochromatosis genotype, was significantly associated with a shorter telomere length in both the crude and adjusted models (Mainous III et al., 2013).

Furthermore, in a subsequent study by Mainous et al. (2014), investigating iron status, telomere length and quality of life in 669 participants, the researchers reported that in the unadjusted model, overall general health ($p < 0.001$) and mental health ($p < 0.01$) statuses were significantly worse among those with elevated transferrin saturation percentage (TS%) ($\geq 50\%$ for men, $\geq 45\%$ for women) compared to non-elevated TS% ($< 50\%$ for men, $< 45\%$ for women). Additionally, telomere length was significantly shorter ($p < 0.01$) in those with elevated TS% compared to the non-elevated TS% subjects. After adjusting for demographics and socio-economic status, those with elevated TS% had poorer mental health status and shorter telomere length compared to the non-elevated TS group, ($p < 0.001$ and $p < 0.01$, respectively). In the fully adjusted model (additional adjustments for chronic disease states), significantly poorer mental health status remained among those in the elevated TS% group ($p < 0.0001$), as was a trend toward shorter telomere length ($p < 0.05$) (Mainous III et al., 2014).

Kozlitina and colleagues investigated the association of parameters of red blood cell size and telomere length in a cohort of 3,157 individuals (Kozlitina & Garcia, 2012). After adjusting for potential confounding variables (age, gender, and ethnicity), the investigators found a strong, significant association between the size of the red blood cell measured by mean corpuscular volume (MCV), as well as the RBC distribution width (RDW), and a shorter mean telomere length ($p < 0.0001$, $p < 0.0001$, respectively). In

addition, they observed that higher hemoglobin levels were also associated with shorter telomere length ($p=0.0009$) in this population. However, contrary to the findings by Mainous et al. (2014), the authors did not find an association between telomere length and iron stores. Although the authors do not speculate, it is well known that iron, as well as, vitamin B₁₂ and folate are key micronutrients involved in red blood cell (RBC) production. Thus, higher hemoglobin could potentially correlate with elevated iron in the erythrocyte, just as elevated MCV and RDW correlate with vitamin B₁₂ and folate deficiency (Kepinska et al., 2015).

It is well understood that an insufficiency of vitamin B₁₂ and/or folate can result in macrocytic red blood cells, identified hematologically by elevated MCV and RDW. While the authors reported an association between elevated MCV, greater RDW and a shorter telomere length, they did not investigate the status of other micronutrients involved, such as vitamin B₁₂ and folate (Kozlitina & Garcia, 2012). Interestingly, homocysteine levels were higher in the shortest tertile of telomere length compared to the longest ($p=0.0054$); however, after full adjustment, the association lost significance. Homocysteine is known to be elevated in both folate and vitamin B₁₂ deficiency. This study demonstrated strength in sample size, although it was limited by design having originated as a cross sectional study in 2000, and later transformed to a longitudinal study in 2007 (Kozlitina & Garcia, 2012).

3. Factors in One-Carbon Metabolism

a. Overview

The requirement of vitamin B₁₂ for folate metabolism has been well established. Vitamin B₁₂ in the form of methylcobalamin serves as a coenzyme in the conversion of homocysteine to methionine (Fig.1). The formation of methylcobalamin requires a methyl group from folate in the form of 5-methyl tetrahydrofolate (5-MTHF). The enzyme Methionine Synthase bound to cobalamin picks up the methyl group from 5-MTHF forming methylcobalamin and tetrahydrofolate (THF). In this process, the methyl group from the newly formed methylcobalamin is transferred to homocysteine converting it into methionine. The formation of 5-MTHF is irreversible, thus a deficiency in vitamin B₁₂ traps folate in this methylate form, commonly referred to as the “methyl-folate trap.” With adequate vitamin B₁₂ status, folate as THF, is available as a substrate for its other coenzyme forms such as, 10-formyl THF which is required for purine synthesis, and 5,10-methylene THF required for thymidylate synthesis essential for DNA synthesis (Gropper, Smith, & Groff, 2009b).

Homocysteine (Hcy) is a non-protein forming amino acid that acts as a methyl carrier in folate metabolism. After synthesis, methionine is activated by ATP to form S-adenosylmethionine (SAMe) which serves primarily as a universal methyl donor in numerous reactions throughout the body. The post-methylation byproduct, S-adenosylhomocysteine (SAH), is subsequently hydrolyzed to Hcy. Homocysteine is then ready to accept another methyl group from the Methionine Synthase–Vitamin B₁₂ complex and repeat the cycle. Via a transsulfuration pathway, catalyzed by cystathionine β-synthase requiring pyridoxal-5'-phosphate (PLP), homocysteine irreversibly condenses

with serine to form cystathionine which is further hydrolyzed by a second PLP-dependent enzyme, gamma-cystathionase, to form cysteine and α -ketobutyrate.

Elevated blood Hcy, or hyperhomocysteinemia, has long been considered a risk factor for deleterious health outcomes, particularly cardiovascular disease (Reynolds, 2006). Much research on this subject culminated in a prominent meta-analysis in which included 27 studies involving over 4,000 patients with occlusive vascular disease (including cardiovascular, peripheral, and cerebrovascular), and over 4,000 healthy controls (Boushey, Beresford, Omenn, & Motulsky, 1995). Data showed that Hcy was an independent risk factor for atherosclerotic vascular disease, specifically, of the coronary, cerebral, and peripheral vessels.

Subsequent studies have gone on to further demonstrate an indisputable link between elevated Hcy and an increased risk of disease. One study out of the Framingham Heart Study showed that among 1933 elderly subjects, those with elevated Hcy (upper quartile) had a greater than two-fold increase in all-cause cardiovascular disease mortality compared to those with lower Hcy levels (lower three quartiles) (RR = 2.18; 95% CI: 1.86, 2.56) (Bostom et al., 1999). The association attenuated but remained significant after adjusting for multiple factors including age, sex, systolic blood pressure, smoking, diabetes, and cholesterol levels (RR = 1.54; 95% CI: 1.31, 1.82) (Bostom et al., 1999).

b. Folate, Vitamin B₁₂, and Homocysteine

As previously discussed, vitamin B₁₂ is a key nutrient in the methylation of homocysteine to methionine, and therefore plays an essential role in the reduction of Hcy levels. With the substantial reduction of folate deficiency following folic acid

fortification, it remains plausible that prevalent cases of hyperhomocysteinemia are related primarily to vitamin B₁₂ deficiency, particularly among the elderly who are at the greatest risk of deficiency due to the higher incidence of pernicious anemia and malabsorption with increased age.

In a prospective study of 1096 older Latino adults shortly after fortification with folic acid was in full operation, hyperhomocysteinemia was present in 181 subjects (16.5%) (Green & Miller, 2005). Of the 181 subjects, 55 (30.4%) had low total vitamin B₁₂ and/or low holotranscobalamin (holoTC). HoloTC is a vitamin B₁₂/carrier protein complex and used as a biomarker of B₁₂ status. Thus, holoTC represents the biologically active fraction of vitamin B₁₂ in the blood, suggesting it may be a more sensitive biomarker for the vitamin than serum levels alone. Among these 55 participants, 29 subjects were low in both vitamin B₁₂ and holoTC. In contrast, only two (1.1%) of the 181 participants with hyperhomocysteinemia had low RBC folate. Of these two subjects, both also had low vitamin B₁₂ and/or low holoTC. According to the study, there was an 8-fold risk of hyperhomocysteinemia because of low vitamin B₁₂ (OR=8.3, 95% CI: 4.7-14.6) and a population attributable risk (PAR%) of 29.7%. The odds for hyperhomocysteinemia associated with low RBC folate was negligible and nonsignificant (OR=1.5, 95% CI: 0.2-11.2) and a PAR% of 0.3. Thus, it can be suggested that in the age of folic acid fortification, hyperhomocysteinemia is more attributable to vitamin B₁₂ deficiency (29.7–36.4%) rather than a deficiency in folate (0.3%) (Green & Miller, 2005).

National Health and Nutrition Examination Survey (NHANES) data from 10,413 participants were analyzed for serum vitamin B₁₂ and folate concentrations, along with

Hcy and MMA (Selhub, Morris, & Jacques, 2007). Prevalence of vitamin B₁₂ deficiency (defined as <148 pmol/L) was 1.6% to 2.2% in the NHANES III and 1999-2000 and 2001-2002 cycles, respectively. The mean Hcy level decreased across increasing folate categories among those with sufficient vitamin B₁₂ levels (\geq 148 pmol/L) in both NHANES surveys. Contrarily, the mean Hcy level for those who were vitamin B₁₂ deficient increased significantly across the four serum folate categories. Furthermore, the mean Hcy for the B₁₂ deficient group with serum folate >32.6 nmol/L was nearly 12 μ mol/L, a commonly used cutoff point for hyperhomocysteinemia. In addition, among those with vitamin B₁₂ deficiency in the NHANES 1999-2002 data, serum MMA concentrations increased significantly across all increasing categories of folate. A significant decrease in MMA was observed in subjects replete in vitamin B₁₂ (>148 pmol/L) with increasing concentrations of folate (Selhub et al., 2007).

Elevated Hcy is indicative of impaired methionine synthase activity secondary to vitamin B₁₂ deficiency, whereas high MMA levels reflect compromised methylmalonyl-CoA mutase activity. These data conclude that both pathways of vitamin B₁₂ metabolism are adversely impacted by elevated folate concentrations (Selhub et al., 2007). Therefore, these data suggest that the enzymatic functions of vitamin B₁₂ deteriorate as serum folate concentrations increase, resulting in elevated MMA and Hcy, the later which is known to be detrimental to health and well-being (Selhub et al., 2007).

Data from the NHANES 1999-2000 and 2001-2002 cycles yielded an association between folate and B₁₂ status in relation to anemia (n=1458) and cognitive impairment (n=1302) in older adults (Morris, Jacques, Rosenberg, & Selhub, 2007). The researchers defined low B₁₂ status as a serum B₁₂ <148 pmol/L or a serum MMA concentration of

>210 nmol/L. High folate status was defined as folate concentrations >59 nmol/L.

Within the study population, nearly 3% were B₁₂ deficient (serum B₁₂ <148 pmol/L), however, 25% met the study criteria for low B₁₂ status. High serum folate concentrations accounted for 4% of the study population.

In the fully adjusted model, participants with normal B₁₂ status and high serum folate had a lower prevalence of anemia (OR: 0.6; 95% CI: 0.2, 2.4) and cognitive impairment (OR: 0.5; 95% CI: 0.2, 0.96) compared to those with normal status in both nutrients. Among those with low B₁₂ status, those with normal serum folate levels had an almost 2-fold increased prevalence of anemia (OR: 1.9; CI: 1.01, 3.6) and a trend toward a 60% increased risk of cognitive impairment (OR: 1.6; CI: 0.95, 2.8) compared to those with normal status in both nutrients. Moreover, among those with low B₁₂ status and high serum folate, the risk of anemia and cognitive impairment increased nearly five-fold (OR: 4.8; 95% CI: 2.3, 10.4; OR: 4.9; 95% CI: 2.6, 9.2, respectively) compared to those with normal status in both vitamins (Morris et al., 2007).

The authors concluded that compared to normal status for both nutrients, high serum folate status alone was associated with a protective effect for anemia, as well as a significant reduction in the prevalence of cognitive impairment. On the other hand, low B₁₂ status regardless of folate status, was associated with a significantly increased risk of anemia and cognitive impairment. Furthermore, the combination of low B₁₂ status and elevated serum folate significantly magnified the risk of both conditions (Morris et al., 2007).

In addition, while elevated homocysteine levels were present in the low B₁₂ / high folate subjects, the prevalence of hyperhomocysteinemia (>13 μmol/L) in that group was

significantly lower than that of the group with low B₁₂ status / normal folate (<13 μmol/L) ($p < 0.001$). Hyperhomocysteinemia primarily due to low B₁₂ status, was associated with an increased risk of both anemia (OR: 2.0; 95% CI: 1.01, 3.8) and cognitive impairment (OR: 1.9; 95% CI: 1.1, 3.4) in this study (Morris et al., 2007).

4. Factors in One-Carbon Metabolism and Health Outcomes

a. Homocysteine and Cognitive Function

Of unique interest, is the association between hyperhomocysteinemia and cognitive decline and dementia. In a prospective study looking at homocysteine levels and risk of dementia and Alzheimer's disease (AD), participants (n = 1092) from the Framingham Heart Study were followed for eight years. Of the 111 subjects who developed dementia, 83 were diagnosed with AD. In the quartile-specific analysis, with the first quartile of Hcy concentration as the reference, the RR of AD was 1.2 (95% CI: 0.6, 2.2) in the second quartile, 1.3 (95% CI: 0.6, 2.5) in the third quartile, and a RR of 2.2 (95% CI: 1.2, 4.1) in the fourth quartile. In addition, plasma Hcy concentrations greater than 14 μmol/L, nearly doubled the risk of AD. Moreover, addition of folate, vitamin B₁₂, and vitamin B₆ status to the model did not significantly alter the association, suggesting Hcy level is a strong, independent predictor of developing AD.

In a recent longitudinal study, researchers investigated the associations between serum Hcy, vitamin B₁₂ and folate on cognition in the elderly (Hooshmand et al., 2012). Measurements were taken at baseline in 274 dementia-free participants living in Finland in 1998. Participants were between the ages of 65 and 79 years of age at the time of entry. Blood samples were analyzed for total Hcy, serum folate and holoTC, as a biomarker of

vitamin B₁₂ status. In addition to the biochemical measurements, several neuropsychological tests were performed to assess cognitive function, including the Mini-Mental State Examination (MMSE), immediate word recall test, the Stroop test, category fluency test, and the bimanual Purdue Pegboard test. These exams provided data which were used to assess global cognition, episodic memory, executive function, verbal expression, and psychomotor speed both at baseline and at follow up.

Hooshmand et al. (2012) found that elevated Hcy was significantly inversely correlated with serum folate ($r = -0.42$) and holoTC ($r = -0.46$), suggesting that high levels of Hcy in this study were likely indicative of low folate and low vitamin B₁₂ status. After adjusting for age, sex, education, follow up time, BMI, blood pressure, smoking status, APOE-4, and presence of renal insufficiency, elevated Hcy was significantly associated with declined cognitive function in all areas assessed. With additional adjustments, the association between Hcy and psychomotor speed lost significance. When adjustments included serum folate and holoTC, the association between Hcy and MMSE score also lost significance.

Elevated holoTC at baseline was significantly associated with greater performance in global cognition (RD = 1.08, 95% CI: 1.00, 1.17), executive function (RD = 1.13, 95% CI: 1.02, 1.25), and psychomotor speed (RD = 1.18, 95% CI: 1.05, 1.23) at follow up. When Hcy and folate were included in the model, the trend toward higher holoTC and improved cognitive performance remained; however, it was no longer significant. No significant associations between serum folate and the areas of cognitive function measured were identified (Hooshmand et al., 2012).

Strengths of this study included the population-based study design and seven-year follow up time, as well as the use of holoTC as a biomarker of vitamin B₁₂ status.

Limitations included small sample size, availability of serum samples restricted to baseline collection only, and the lack of other biomarkers for both vitamin B₁₂ and folate status, including MMA and RBC folate.

In a recent systematic review and meta-analysis of modifiable risk factors associated with cognition and dementia, investigators included 247 original epidemiologic studies (167 cohort studies, 80 cross-sectional studies) in their review (Beydoun et al., 2014). Risk factors included smoking, education, alcohol intake, physical activity, and Hcy, as well as nutritional factors including caffeine intake, omega-3 fatty acids, and antioxidants. Overall, 19 cohort studies investigated Hcy and cognitive function. Of the 19 studies, 12 found elevated serum Hcy levels to be associated with poorer cognitive function and/or dementia. Two studies found an association with Hcy and some cognitive outcomes, but not others, and five studies were unable to detect a significant association with cognitive function (Beydoun et al., 2014). Homocysteine is said to have neurotoxic and excitotoxic properties *in vitro*, proposing a direct effect on cognitive function (Kruman et al., 2000).

Homocysteine exposure to the primary hippocampal neurons in cultured cells resulted in apoptosis in a dose-dependent manner (Kruman et al., 2000). DNA strand breaks occurred rapidly in the neurons exposed to Hcy. Neuronal cells appeared to be more vulnerable to the effects of Hcy-induced apoptosis compared to other cells including astrocytes and cultured vascular endothelial cells. Hcy also appears to render neural cells more vulnerable to oxidative stress and excitotoxicity. The investigators

tested their hypothesis *in vivo* by infusing Hcy, alone and with a seizure-inducing excitotoxin called kainite, into the hippocampus of adult mice. While Hcy alone did not damage the hippocampal neurons, the combination markedly exacerbated kainite-induced damage to the pyramidal neurons (Kruman et al., 2000). These and other data demonstrating that Hcy can sensitize neurons to such adverse conditions, provide insight into how Hcy might contribute to the pathogenesis of neurodegenerative disorders and moreover suggest a possible mechanism by which nutrients involved in one-carbon metabolism may reduce the risk of such disorders.

b. Folate and Cognitive Function

Several studies have investigated the association of serum folate and cognitive decline (Duthie et al., 2002; Kado et al., 2005; Ramos et al., 2005; Ravaglia et al., 2005). Ramos et al. examined the association of folate status and cognitive function and dementia in 1789 community-dwelling elderly Latinos (Ramos et al., 2005). Multiple linear regression analyses were used to describe the associations between RBC folate concentration and cognitive function tests in four models before and after adjustment for serum Hcy, vitamin B₁₂, serum creatinine, and demographics. Cognition was assessed via six measurements including the Modified Mini-Mental State Examination (3MSE), delayed recall, object naming, picture association, verbal conceptual thinking, verbal attention, and pattern recognition. In addition, odds ratios were evaluated as indicators of the relationship between RBC folate and serum Hcy as independent variables and diagnostic measures of cognitive decline and dementia. After adjusting for serum Hcy alone, RBC folate remained inversely correlated with six of the seven cognitive tests. The

analysis indicated that RBC folate explained 4.0–6.1% ($R^2 = 0.040–0.061$) of the variance in cognition function scores within the sample. The addition of serum vitamin B₁₂ and creatinine did not significantly influence the results. However, adding the demographic variables (age, sex, education, and acculturation) and Center for Epidemiological Studies Depression Scale (CES-D) score to the model significantly attenuated the associations. Only the 3MSE and delayed recall remained significant ($p=0.005$ and $p=0.007$, respectively) (Ramos et al., 2005). When evaluating RBC folate in the secondary analysis, the odds ratio (OR) for low 3MSE (OR = 0.51, 95% CI: 0.32, 0.83) and dementia diagnosis (OR = 0.28, 95% CI: 0.11, 0.72) were significant after adjusting for all potential confounders. These data indicate that risk of cognitive impairment and dementia decreased with elevating concentrations of RBC folate (Ramos et al., 2005).

Folic acid fortification of grains beginning in the late 1990s has significantly reduced the prevalence of folate deficiency in the United States (defined as plasma folate <6.8 nmol/L) from 22% to approximately 1.7% of the population, and the prevalence of hyperhomocysteinemia by almost one-half (Jacques, Selhub, Bostom, Wilson, & Rosenberg, 1999). Despite folic acid fortification, hyperhomocysteinemia (defined as >13 $\mu\text{mol/L}$) is still found in approximately 9% of the U.S. population, likely due to poor B₁₂ status, and remains a key risk factor for adverse health outcome.

c. Vitamin B₁₂ and Cognitive Function

Despite the association of low vitamin B₁₂ status with hyperhomocysteinemia, and the later with cognitive decline, few studies have demonstrated an independent

association between vitamin B₁₂ and impaired cognition. In an early study, researchers investigated the neuropsychiatry of megaloblastic anemia to distinguish which symptoms were associated with vitamin B₁₂ deficiency versus folate deficiency (Shorvon, Carney, Chanarin, & Reynolds, 1980). Prior to this, no comprehensive study had been performed on the neuropsychiatry of vitamin B₁₂ deficiency since the early 1950s. Shorvon et al. compared the neurological and psychiatric states of 84 patients admitted to a local London hospital. Inclusion criteria included megaloblastic anemia associated with a serum vitamin B₁₂ of less than 150 ng/L (110.7 pmol/L) or a serum folate below 2.5 µg/L (5.7 nmol/L) together with an RBC folate level below 150 µg/L. Patients with evidence of both deficiencies, and/or diseases known to impact the metabolism of these nutrients were excluded from the study. Seventy-seven of the 84 patients underwent an electrophysiological study to identify peripheral nerve function (Shorvon et al., 1980).

Fifty patients (59%) were diagnosed with vitamin B₁₂ deficiency and 34 (41%) had confirmed folate deficiency. The ratio of males to females in each group was similar, although the mean age was higher in the vitamin B₁₂ deficient group (mean age: 60 years) compared to the folate deficient group (mean age: 40 years). Of the 50 patients with vitamin B₁₂ deficiency, 32 had pernicious anemia. Hematological data showed no significant differences between the two groups with respect to hemoglobin and mean cell volume (MCV). Approximately two-thirds of both groups had some form of neuropsychiatric abnormality. Among the vitamin B₁₂ deficient group, 20 patients (40%) had peripheral neuropathy compared to only 6 patients (18%) of the folate deficient group. In addition, eight patients with vitamin B₁₂ deficiency had subacute combined cord degeneration. This condition affects the spinal column, brain and peripheral nerves

first, by damage to the myelin sheath, and later, damage to the nerve cell itself. Symptoms can include paresthesia, unsteady gait, depression, visual changes, and changes in mental status, including cognitive decline and dementia. There were no cases of subacute combined cord degeneration in the folate deficient group.

Further findings showed 28 patients (65%) with vitamin B₁₂ deficiency demonstrated peripheral nerve disease based on electrical evidence, while only 6 patients (21%) with folate deficiency reported the abnormal outcomes. The authors reported that the clinical and electrical evidence suggests that neuropathy is approximately three times more common in vitamin B₁₂ deficiency than in folate deficiency. While this study was not designed to detect changes in cognitive decline, it alludes to the potential, particularly with subacute combined cord degeneration observed only in the patients with vitamin B₁₂ deficiency (Shorvon et al., 1980).

In a randomized placebo-controlled study, researchers investigated the effects of B-vitamin supplementation on Alzheimer's disease (AD) related gray matter atrophy over a two-year period in 156 elderly participants (Douaud et al., 2013). Eighty subjects (mean age 77 years) received the B-vitamin mixture (0.8 mg/d of folic acid, 0.5 mg/d of vitamin B₁₂, 20 mg/d of vitamin B₆) and 76 subjects (mean age 76 years) received a placebo. There were no significant differences in demographics, cognitive function, or biomarkers measured at baseline between the two groups.

Local gray matter volume was assessed at baseline and again after 2 years via MRI scan. Atrophy was found in similar areas in both groups; however, the group receiving the B-vitamins showed a significant reduction of atrophy in the posterior regions of the brain, those most affected by AD (Douaud et al., 2013).

The average reduction of gray matter over the two-year period was 3.7% (± 3.7) in the placebo group and 0.5% (± 2.9) in the B-vitamin group. Figure 1 shows the loss of gray matter volume (shown in yellow) for both groups. The amount of yellow corresponds to the volume of loss.

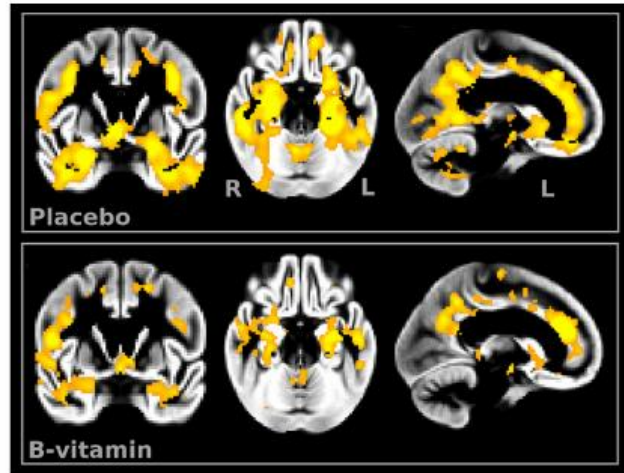


Fig 1: Regional loss of gray matter volume. Douaud et al., 2013

To investigate the effect of serum Hcy on gray matter decline with and without treatment, the researchers dichotomized each group by serum Hcy level (those above and those below the median of 11.06 $\mu\text{mol/L}$). Within the placebo group, participants with higher serum Hcy had more gray matter atrophy compared to those with lower Hcy concentrations. In the treatment group, the researchers found no difference in atrophy between the two Hcy groups. When testing the effect of B-vitamin supplementation on overall atrophy, the authors found no effect of the treatment in participants with low baseline serum Hcy levels. In the higher serum Hcy group however, B-vitamin supplementation had a protective effect on gray matter loss. Those with high serum Hcy showed reduced gray matter atrophy after two years of B-vitamin treatment. The average loss of gray matter over a two-year period for those with high serum Hcy in the placebo group (n=35) was 5.2% (± 3.4) compared to those with high serum Hcy in the B-vitamin group (n=42) who showed an average loss of 0.6% (± 2.1) (Douaud et al., 2013).

The researchers state that the reduced atrophy in regions of the brain susceptible to AD appeared to be mediated by lowering of serum Hcy levels. Furthermore, while the

B-vitamin treatment led to a change in plasma vitamin B₁₂ and plasma folate concentrations, it was only plasma vitamin B₁₂ that appeared to play a role in modifying Hcy levels. The lack of influence of serum folate may be due to the fact these participants had good folate status (mean folate 29 nmol/L) at baseline. No participant was found to be vitamin B₁₂-deficient (based on the cutoff level of <150 ng/L). Nonetheless, changes in gray matter atrophy were significantly associated with a change in plasma vitamin B₁₂ levels. Elevated serum Hcy is an inhibitor of both methylation reactions and the proliferation of neuronal cell precursors. Vitamin B₁₂ is responsible for methylation in the brain, and its deficiency can disrupt neuronal cell proliferation. Thus, it is plausible that vitamin B₁₂ is essential in maintaining adult neurogenesis in the regions of the brain affected by AD (Douaud et al., 2013).

d. Vitamin B₁₂ and Type 2 Diabetes

Neuropathic manifestations from microvascular complications are highly associated with diabetes. Vitamin B₁₂ deficiency can exacerbate existing neuropathic conditions in people with type 2 diabetes (T2DM) (Adaikalakoteswari et al., 2014). Metformin, a class of insulin sensitizing drugs called biguanides, is the most commonly prescribed oral hypoglycemic agent in patients with T2DM (Liu et al., 2009). Use of metformin is well known to impede the absorption of vitamin B₁₂ resulting in a deficiency; however, it is often overlooked and seldom investigated. Researchers sought to define the prevalence of vitamin B₁₂ deficiency in a T2DM population within a military primary care practice (Pflipsen et al., 2009). Men and women (n=195) 45 years of age and older, with a diagnosis of T2DM were included in the study. Vitamin B₁₂

deficiency was defined as a plasma B₁₂ level <100 pg/mL (<74 pmol/L), or a plasma B₁₂ level of 100-350 pg/mL (74 – 258 pmol/L) with a corresponding methylmalonic acid (MMA) level >243 nmol/L and/or a homocysteine (Hcy) level >11.9 nmol/L. Inclusion of alternative biomarkers of B₁₂ status have been shown to improve the sensitivity and specificity of detecting a deficiency and may help to identify early, asymptomatic cases of B₁₂ deficiency before irreversible neurological damage. Only 60% of those with vitamin B₁₂ deficiency have clinical manifestations of macrocytic anemia; thus, additional diagnostic measures are essential (Stabler, 2013).

Serum vitamin B₁₂ levels ranged from 91 to 2818 pg/mL (67 to 2079 pmol/L). Only one subject was deficient based solely on serum B₁₂ <100 pg/mL, while 79 had insufficient levels of serum vitamin B₁₂ of 100-350 pg/mL (74 – 258 pmol/L), 43 proceeded to have either elevated MMA (>244 nmol/L) and/or elevated serum Hcy levels (>11.9 nmol/L) indicating vitamin B₁₂ deficiency. In total 44 participants (22%) with T2DM were B₁₂ deficient. Patients with B₁₂ deficiency had T2DM significantly longer than those who were B₁₂ sufficient (Pflipsen et al., 2009). When a subsequent analysis was conducted defining all participants with serum B₁₂ levels <350 pg/mL (<258 pmol/L), patients using metformin were at significantly greater risk of B₁₂ deficiency. While patients taking metformin had a significantly lower B₁₂ status compared to patients not taking the medication (425.99 pg/mL and 527.49 pg/mL, respectively), metformin use was not significantly associated with B₁₂ deficiency. The authors stated that their inclusion of diagnostic criteria other than serum B₁₂ levels (MMA and Hcy) in defining B₁₂ deficiency likely improved the diagnosis of the condition. However, at the time there

were no published studies examining the prevalence of B₁₂ deficiency utilizing the more sensitive and specific diagnostic markers in the general population.

While this study was innovative, it contained some limitations. The cohort was from a military family medicine center that was deemed to be exceptional in the chronic care of diabetes. Thus, most participants were considered to have well-controlled T2DM, which may not be generalizable to the rest of the population. In addition, this study did not investigate the clinical manifestations of B₁₂ deficiency such as megaloblastic anemia. Analyses of mean corpuscular volume (MCV), red cell distribution width (RDW) and/or hemoglobin and hematocrit (H/H) may have been advantageous in further defining clinical outcomes. Furthermore, the cross-sectional design does not allow for causality; however, the aim of the study was to define vitamin B₁₂ deficiency in a diabetic population, for which the design was appropriate.

Data from the 1999-2006 National Health and Nutrition Examination Survey (NHANES) were published on the association of vitamin B₁₂ deficiency in US adults ≥ 50 years with (n=1,621) and without (n=6,867) T2DM and with and without metformin usage (Reinstatler, Qi, Williamson, Garn, & Oakley, 2012). The authors defined biochemical vitamin B₁₂ deficiency as serum levels ≤ 148 pmol/L, borderline deficiency as >148 to ≤ 221 pmol/L, and normal as >221 pmol/L. Exclusion criteria included pregnancy, HIV+ status, B₁₂ injection therapy, prediabetes, and elevated creatinine levels, but included participants with existing liver and/or renal conditions. The authors examined B₁₂ supplement use, acid blockers, homocysteine levels, MCV, hemoglobin A_{1c} %, and serum folate among participants with and without T2DM. The variables were then stratified by metformin use (yes, n=575; no, n=1,046) within the T2DM group. Of

interest, serum vitamin B₁₂ was significantly lower in participants with T2DM taking metformin compared to those with T2DM not taking the medication ($p=0.0116$), as well as to non-diabetics ($p=0.011$). The weighted prevalence of vitamin B₁₂ deficiency adjusted for age, race, and gender was 5.8% for those with T2DM taking metformin, and 2.2% not taking metformin, and 3.3% in those without T2DM. The investigators applied the Fleiss formula for attributable risk in cross-sectional data and found that among all cases of vitamin B₁₂ deficiency, 3.5% were attributable to metformin use. Among those with T2DM, 41% of the vitamin B₁₂ deficient cases were attributable to the medication usage. Although serum homocysteine levels were investigated, no significant differences were found (Reinstatler et al., 2012).

Interestingly, the researchers noted that despite the observed effects of metformin use on vitamin B₁₂ status, the overall deficiency percentage may not indicate a public health concern. The authors also intentionally did not include MMA as an indicator of vitamin B₁₂ status. They concluded that the use of MMA and other functional measures of vitamin B₁₂ status remains controversial. However, findings from a roundtable discussion on measurement of vitamin B₁₂ status biomarkers in NHANES data revealed that measuring a circulating biomarker such as serum vitamin B₁₂ or holoTC and a functional biomarker such as MMA or Hcy is preferable to measuring only one biomarker in NHANES (Yetley et al., 2011). In addition, the panel concluded that if NHANES reinstates measurement of vitamin B₁₂ status in the future, the biomarkers will be serum vitamin B₁₂ and plasma MMA.

5. Folate, Vitamin B12 and Telomere Length

As two important factors in one-carbon metabolism, vitamin B₁₂ and folate status vary in their association with telomere length. In most reports, vitamin B₁₂ has shown to have no association with telomere length, while studies investigating a relationship between folate levels and telomere length appear to be more complex. One study looked at the association between folate status and the 677C>T polymorphism of the MTHFR gene and telomere length (Paul et al., 2009). The authors controlled for vitamin B₁₂ in their analyses, but also reported that plasma vitamin B₁₂ concentration was not associated with telomere length in their population of 195 healthy men. Interestingly, the authors reported a U-shaped relationship between plasma folate status and telomere length, with longer telomeres associated with both low and high folate status. The investigators reported that as plasma folate concentration moved toward the median (11.6 nmol/L), there was a trend toward a corresponding decrease in mean telomere length. In those with folate concentrations below the median, there was a corresponding increase in telomere length. Thus, the relative telomere length of men in the lowest quartile of plasma folate concentration tended to be higher than the telomere length of men in the highest quartile of plasma folate concentration ($p = 0.11$). The authors suggested that longer relative telomere length observed among those with very low folate status may be a result of telomeric dysregulation due to hypomethylation of DNA (Paul et al., 2009).

This finding was supported by a study utilizing an *in vitro* model of chronic folate deficiency (Bull et al., 2014). Cells were grown under three simulated conditions: deficient, replete, and/or supraphysiological. The folate-deficient cells had a significantly longer mean telomere length at day 7 and 14 compared to the other two cell conditions at

the same time points ($p=0.02$). However, the mean telomere length dropped sharply after day 14 to below that of the other two cell conditions by day 42, indicating an increase in telomere length with the beginning of folate deficiency (days 7–14), followed by a shortening of telomere length with chronic deficiency (days >14).

In a previous study, the authors investigated the relationship of plasma folate, homocysteine, vitamin B₁₂ and RBC folate to telomere length in a fairly homogeneous population of 90 predominantly Caucasian, healthy men and women (Bull, O’Callaghan, Mayrhofer, & Fenech, 2009). In a dichotomous grouping of younger (18-32 years) and older (65-83 years) participants, the authors reported the mean plasma folate and RBC folate concentrations were significantly higher in the older subjects compared to the younger cohort ($p = 0.0001$ for both); however, no correlations were observed between plasma and/or RBC folate and telomere length, nor with plasma vitamin B₁₂ and telomere length in this study. In addition, no differences were observed in the plasma vitamin B₁₂ concentrations between the two cohorts. Of note, this study was limited in sample size and likely lacked sufficient power to detect a significant finding if indeed one existed (Bull et al., 2009).

Despite the above findings, current research suggests vitamin B₁₂ deficiency is more common in older adults. Vitamin B₁₂ deficiency ranges from 5–40% (depending on the definition used) resulting from inadequate vitamin B₁₂ intake and more commonly, impaired absorption (Wong, 2015). According to NHANES data, the prevalence of vitamin B₁₂ deficiency (serum B₁₂ <148 pmol/L) among those 20–39 years of age was <3%, and close to 4% for those aged 40–69 years (Allen, 2008). For individuals over 70 years, the prevalence was double that of the younger group at 6%. Among those

considered to be marginally deficient (serum B₁₂ of 148–221 pmol/L), the percentage increased to 14%–16% in those aged 20–59, to over 20% in those over 60 years old. In other parts of the world, prevalence of deficient or marginal vitamin B₁₂ status among children and adults can range from 40% in Latin America to as high as 70% - 80% in Africa and India, respectively (Allen, 2008).

In a seminal publication by Liu et al. investigating factors of one-carbon metabolism from the Nurses' Health Study (NHS), the authors reported no associations between plasma folate ($P_{\text{-trend}} = 0.90$), plasma vitamin B₁₂ ($P_{\text{-trend}} = 0.94$), plasma vitamin B₆ (PLP) ($P_{\text{-trend}} = 0.78$), and plasma homocysteine levels ($P_{\text{-trend}} = 0.57$) and relative telomere length in a total of 1,624 participants, after adjusting for age, smoking, body mass index (BMI), and physical activity (Liu et al., 2013). Higher plasma cysteine levels were significantly associated with shorter relative telomere length in this population ($P_{\text{-trend}} = 0.02$). Furthermore, the authors reported no significant associations found among the dietary intakes of folate ($P_{\text{-trend}} = 0.94$), vitamin B₁₂ ($P_{\text{-trend}} = 0.55$), and vitamin B₆ ($P_{\text{-trend}} = 0.71$) and quartiles of relative telomere length after adjusting for the potential confounders (J. J. Liu et al., 2013).

The authors noted that this was the only study investigating all factors of one-carbon metabolism on telomere length. While this study demonstrated strength in sample size and use of physiological biomarkers of vitamin B₁₂ and folate, it has some limitations. The study was cross-sectional by design, which negates the ability to make causal inference. In addition, the study was conducted predominately in white women. It remains possible an association exists in men or in different racial/ethnic groups. Finally, the only representation of vitamin B₁₂ and folate status were plasma levels of the

vitamins. The authors did not investigate MMA as a biomarker of vitamin B₁₂ status which has been reported in literature to be superior to plasma vitamin B₁₂ levels in defining total body status. Additionally, the authors did not investigate RBC folate, which has been suggested to be a better representation of current folate status.

Data from the Framingham Offspring Cohort (1991-1995 and 1995-1998) yielded an association between plasma folate levels post-folic acid fortification and telomere length (Paul et al., 2014). The authors controlled for covariates including age, sex, BMI, smoking, alcohol intake, serum creatinine, and menopause status in 1,044 men and women. General linear regression models were used to determine the association between telomere length and plasma folate in quintiles, as well as for folic acid fortification and supplement use. The mean telomere length of those participants examined before folic acid fortification was 6.95 kb (± 0.04), while individuals examined post-fortification had a mean telomere length of 6.88 kb (± 0.04) ($p = 0.14$) (Paul et al., 2014). When plasma folate concentrations were divided into quantiles, those in the fifth quintile (highest 20% of serum folate levels) had a significantly shorter mean telomere (-180 bp) length compared to those in the second quintile ($p < 0.01$) (Paul et al., 2014). While not significant, individuals in the first quintile of plasma folate had a shorter mean telomere length compared to those in the second quintile, revealing a non-linear, bell-shaped relationship. These data coincide with previous literature supporting the relationship between folate and markers of health status. Low plasma folate is associated with DNA hypomethylation and telomere length is determined by DNA integrity. Loss of DNA and histone methylation, for which folate plays a major role, results in a dysregulation, yielding unusually long, dysfunctional telomeres in cell culture (Bull et al., 2014). Thus,

low, as well as elevated plasma folate, can impair DNA synthesis and adversely affect telomere length. Extreme plasma folate concentrations have been associated with poorer health outcomes, and in this study, a shorter mean telomere length (Ericson et al., 2010; Paul et al., 2009).

Data were collected both pre- and post-folate fortification. While the mean telomere length of participants was shorter post-fortification, the difference was not significant (Paul et al., 2014). The investigators also looked at supplement usage. Multivitamin (MVI) users had a significantly shorter telomere length compared to non-users ($p = 0.015$). This finding contrasts with that of Xu et al., who found a positive correlation between MVI use and telomere length (Xu et al., 2009). Interestingly, 83% of those in the fifth quintile of plasma folate (shortest telomere length) were multivitamin users. Increasing plasma folate concentration was associated with a shorter mean telomere length (second through fifth quintiles) even after multivitamin users were removed from the analysis ($P_{\text{trend}} < 0.01$). Additionally, while plasma folate concentration was negatively associated with plasma homocysteine levels ($r = -0.2426$, $p < 0.0001$), the investigators found no association between serum vitamin B₁₂ and/or serum pyridoxal 5'phosphate (PLP) and telomere length (Paul et al., 2014).

Although the study by Xu et al. (2009) found an association between elevated plasma folate and shorter telomere length, other studies have found a positive relationship between the two variables. In a population-based cohort, investigators measured homocysteine and serum folate levels in 1,319 healthy subjects (91.5% women) (Richards et al., 2008). The authors reported higher serum folate levels were associated with an increase in age-adjusted telomere length ($\beta = 0.01$, 95% CI: 0.004-0.017)

(Richards et al., 2008). Homocysteine levels were found to be inversely correlated with telomere length after adjusting for age, C-reactive protein (CRP) levels, physical activity, and glomerular filtration rate (GFR) ($P_{\text{trend}}=0.007$). When serum folate was dichotomized (high and low) within tertiles of homocysteine concentration, the difference in telomere length between the high folate group within the lowest tertile of homocysteine and the low folate group within the highest tertile of homocysteine was 159 bp (95% CI: 64 -255; $p = 0.001$). According to the researchers, this is equivalent to 8.6 years of biological aging. Within each tertile of homocysteine, individuals with higher serum folate levels had a longer mean telomere length ($P_{\text{trend}}=0.002$).

Similarly, researchers found that higher plasma folate status in older men was associated with longer telomere length ($p = 0.04$) (Bull et al., 2009). However, this association was not observed in older women, or in the younger cohort. The authors also reported a significant association between higher homocysteine levels and shorter telomere length in older men ($p = 0.004$). Likewise, Rane et al. found a significant association between elevated Hcy levels and shorter telomere length in their study of 100 adult men and women randomly selected from a prospective population-based cohort of 63,257 Chinese citizens ($P_{\text{trend}} = 0.014$) (Rane et al., 2015). While mean telomere length was longest among those in the highest tertile for folate concentration, the association did not reach statistical significance.

D. Summary

As one of the common hallmarks of aging, telomere attrition is associated with morbidity and mortality. Use of telomere length as a biomarker of health and aging is gaining credence in epidemiological studies. Shorter telomeres have been associated with

many chronic health outcomes such as cardiovascular disease, Alzheimer's disease, cancer, and diabetes. Furthermore, telomere length has also been shown to be an independent risk factor and predictor for insulin resistance, type 2 diabetes, as well as cardiovascular disease related events including myocardial infarction and stroke.

Micronutrients are vital components for proper body functioning; however, in excess, these essential compounds can be detrimental toward optimal health. To date, evidence of a causal relationship between poor iron status and/or factors of one-carbon metabolism and telomere length remains elusive. Of the studies performed, many have been potentially underpowered, or lacked sufficient biomarkers for proper categorization of exposure variables.

The relationship between folate and telomere length thus remains controversial, with evidence of both non-linear, linear, and no associations found (Bull et al., 2009; Paul et al., 2009, 2014; Richards et al., 2008). It remains unclear as to whether there is an ideal folate status at which telomere length is optimal. Furthermore, while no studies have demonstrated a direct association with vitamin B₁₂ status and telomere length, to our knowledge, they have not used MMA as an additional biomarker of vitamin B₁₂ status. Evidence suggests vitamin B₁₂ and folate play key roles in factors that may influence telomere length; thus, further investigation on factors of one-carbon metabolism would fill a much-needed gap in the literature.

To our knowledge, no studies have published data on iron deficiency and telomere length; thus, it is unclear whether low iron status or iron deficiency is associated with telomere length. As a public health concern, iron deficiency is the most common nutritional deficiency worldwide and leading cause of anemia in the United States (World

Health Organization, 2001). Although two studies have alluded to the negative effect of high iron status (hemochromatosis) on telomere length (Mainous III et al., 2013, 2014), larger studies in diverse populations are lacking. Hence, research on overall iron status and telomere length is warranted.

E. Objective

The objective of this research project is to investigate the impact of micronutrient status on telomere length. Our central hypothesis is that altered micronutrient status (i.e. low vitamin B₁₂ status, suboptimal and/or elevated folate status, suboptimal and/or elevated iron status) will be associated with a shorter mean telomere length when compared to those with sufficient status of the corresponding micronutrients. There is plausible evidence to suggest the use of telomere length as a biomarker for predicting disease risk and mortality. Our rationale for undertaking this research is that by identifying modifiable nutritional risks associated with shorter telomere length, we may be able to positively impact biological aging and disease risk by correcting the impaired micronutrient status of vulnerable individuals.

1. Papers #1 and #2: Impaired Levels of Select Factors of One-Carbon Metabolism are Associated with a Shorter Mean Telomere Length in Adult Men and Women.

Our *working hypothesis* for this aim is that suboptimal vitamin B₁₂ and folate status are associated with a shorter mean telomere length in adult men and women, independent of one another. Our *experimental approach* will be to investigate the association between vitamin B₁₂ and folate status, determined by serum vitamin B₁₂ concentration and methylmalonic acid (MMA) level for B₁₂ status and serum folate concentration for folate

status, and mean telomere length in base pairs to determine the extent to which suboptimal vitamin B₁₂ status and/or suboptimal or elevated folate status are independently associated with telomere length, in a nationally representative sample of adult men and women in the United States.

a. Specific Aim: Factors of One-Carbon Metabolism Status and Telomere Length

To evaluate the association of vitamin B₁₂ and folate status on telomere length in a nationally representative sample of adult men and women.

Hypothesis 1a: Compared to participants with adequate vitamin B₁₂ status, men and women with suboptimal vitamin B₁₂ status will have a shorter mean telomere length after controlling for relevant covariates including folate status.

Hypothesis 1b: Compared to participants with adequate folate status, men and women with deficient, as well as elevated plasma folate, will have a shorter mean telomere length after controlling for relevant covariates including B₁₂ status.

Hypothesis 1c: Compared to subjects with sufficient vitamin B₁₂ and folate status, men and women with suboptimal vitamin B₁₂ status and elevated folate status will have a shorter mean telomere length after controlling for relevant covariates.

Gap in Knowledge: Previous studies investigating micronutrient status have reported inconclusive results. Studies investigating vitamin B₁₂ have found no association between serum B₁₂ or low vitamin B₁₂ intake and telomere length. One study did find an association with use of vitamin B₁₂ supplements and a longer mean telomere length.

To our knowledge, no study has explored the association of suboptimal vitamin B₁₂ status (defined by serum vitamin B₁₂ and MMA concentrations) and telomere length in a nationally representative, population-based sample. In addition, investigations of

folate status and telomere length have yielded varying results, and the association appears to be complex. Research has demonstrated a non-linear, bell-shaped relationship between plasma folate and mean telomere length, as well as a linear relationship between the two variables. Given the interwoven relationship between folate and vitamin B₁₂ with regards to metabolism and nutrient status, the two micronutrients should be investigated concurrently.

4. Paper #2: Impaired Iron Status is Associated with Shorter Telomere Length in Adult Men and Women.

Our *working hypothesis* for this aim is that low iron status (iron-deficiency anemia and iron deficiency) as well as elevated iron status, are associated with a shorter mean telomere length in adult men and women. Our *experimental approach* will be to investigate the association between iron status, determined by serum iron concentration, serum ferritin, transferrin saturation percent, erythrocyte protoporphyrin level, and hemoglobin concentration, and mean telomere length to determine the extent to which suboptimal iron status is associated with telomere length in a nationally representative sample of adult men and women.

a. Specific Aim: Iron Status and Telomere Length

To evaluate the association of iron status on telomere length in a nationally representative sample of adult men and women.

Hypothesis 2a: Compared to subjects with adequate iron status, men and women with low iron status will have a shorter mean telomere length after controlling for relevant covariates.

Hypothesis 2b: Compared to subjects with adequate iron status, men and women with an elevated iron status will have a shorter mean telomere length after controlling for relevant covariates.

Gap in Knowledge: To our knowledge, no study has investigated the impact of iron deficiency on telomere length in adult men and women. Few studies have examined the role of elevated iron status on telomere length in adults. Of the studies conducted, research has focused on transferrin saturation and genetic markers of hemochromatosis as indicators of iron status. Furthermore, the use of multiple biomarkers of iron status including serum iron, ferritin levels, transferrin saturation percentage (TS%), erythrocyte protoporphyrin, and hemoglobin levels, will provide additional criteria for determining iron deficiency as well as that of elevated iron status or iron overload.

CHAPTER 2

**FACTORS OF ONE-CARBON METABOLISM AND
TELOMERE LENGTH IN ADULT MEN AND WOMEN**

A. Introduction

Telomeres are repetitive, non-coding nucleotide sequences at the end of all vertebrate chromosomes (TTAGGG in humans) first recognized by Muller and McClintock in 1938 and 1941, respectively through their work with *Drosophila* (McClintock, 1941). Telomeres function as protective caps on the chromosomes and play an indispensable role in DNA replication and stability. Credit for this discovery went to Blackburn, Greider, and Szostak, who were awarded the Nobel Prize in Physiology or Medicine in 2009. The guanine-rich 3' projection of the telomere is thought to be of particular importance; acting as a buffer to protect gene coding sequences enabling gene continuity through DNA replication (Greider, 1998). However, the guanine residues of telomeres also make them highly susceptible to damage caused by oxidative stress (Houben, Moonen, van Schooten, & Hageman, 2008; Matthews et al., 2006; Von Zglinicki, 2002).

Telomere attrition has become a widely accepted cellular event associated with aging. Additionally, telomeric loss and dysfunction have been associated with chronic conditions such as cardiovascular disease (Fitzpatrick et al., 2007), hypertension (Samani et al., 2001), type 2 diabetes, (Verhulst et al., 2016; Willeit et al., 2014; Zhao et al., 2014) Alzheimer's disease (Panossian et al., 2003), and certain cancers (Du et al., 2015; Willeit et al., 2010) independent of chronological age. Thus, telomere length assessment holds potential as a biomarker of risk assessment for adverse health outcomes.

It is well known that dietary factors can influence the risk of chronic disease, but the mechanisms of action by which micronutrient status influences chronic disease outcomes are complex and, in most cases, uncertain. Antioxidants can quench reactive oxygen species (ROS) resulting from oxidative stress, and thus may provide protection to susceptible telomeres. Several studies have demonstrated a positive relationship between dietary antioxidant intake and telomere length (Boccardi et al., 2013; García-Calzón, Zalba, et al., 2015; Marcon et al., 2012; Mazidi, Kengne, & Banach, 2017), as well as antioxidant supplementation (Xu et al., 2009). Factors affecting one-carbon metabolism could also influence telomere length; however, findings from studies investigating intake of nutrients involved in this pathway have been inconsistent (Liu et al., 2013).

Folate and vitamin B₁₂ are two important B-vitamins involved in one-carbon metabolism. Xu et al. (2009) reported a longer mean telomere length associated with women who consumed vitamin B₁₂ supplements compared to non-users, although vitamin B₁₂ status was not measured. In most studies, plasma vitamin B₁₂ concentration has not been associated with telomere length, and the association between plasma folate and telomere length has been inconsistent (Bull et al., 2009; Liu et al., 2013; Paul et al., 2014). In previous studies involving telomere length, B₁₂ status had been identified using serum vitamin B₁₂ level as the sole indicator; while the association between plasma folate concentration and telomere length has been reported to be a U-shaped relationship (Paul et al., 2009), a bell-shaped relationship (Paul et al., 2014), as well as having no association (Liu et al., 2013).

Of recent interest are reports of the apparent negative interactions between elevated folate intakes in participants and low B₁₂ status on various health outcomes

(Morris et al., 2007; Morris, Selhub, & Jacques, 2012). Using the NHANES database, researchers investigated the cross-sectional association between serum folate and vitamin B₁₂ status relative to macrocytic anemia and cognitive impairment among 1,459 elderly participants. After controlling for potential confounders, low B₁₂ status (<148 pmol/L and/or MMA >210 nmol/L) was associated with increased risk of anemia (OR 2.7; 95% CI 1.7, 4.2), macrocytic anemia (OR 1.8; 95% CI 1.01, 3.3), and cognitive impairment (OR 2.5; 95% CI 1.6, 3.8) (Morris et al., 2007). Additionally, those with low B₁₂ status and elevated serum folate (>59 nmol/L) had a greater risk of anemia (OR 3.1; 95% CI 1.5, 6.6) and cognitive impairment (OR 2.6; 95% CI 1.1, 6.1) (Morris et al., 2007). Explicitly, anemia and cognitive impairment were observed nearly five-times as often in this group, compared to the group with normal status in both vitamins. In contrast, normal vitamin B₁₂ status (>256 pmol/L) coupled with high folate, provided protection against cognitive impairment (OR 0.4; 95% CI 0.2, 0.9) (Morris et al., 2007).

In a longitudinal study, the same authors further investigated B₁₂ and folate status and cognition in 549 participants using the prospective Framingham Heart Study (FHS) and the Mini-Mental State Examination with an 8-year follow up (Morris et al., 2012). Vitamin B₁₂ and folate status were assessed in quintiles. The average rate of cognitive decline was 0.24 points/year. There was a significantly faster rate of cognitive decline (0.35 points/year) reported among participants in the lowest two quintiles (< 256.8 pmol/L) compared to the upper three-fifths (\geq 256.8 pmol/L) of vitamin B₁₂ status. With folate, the lowest two quintiles and the upper most quintile had similar and faster rates of cognitive decline, compared to the third and fourth quintiles. This parallels data on folate status and telomere length in which the lowest and highest concentrations of folate have

negative outcomes. Furthermore, in the presence of low B₁₂ status, high plasma folate (≥ 20.2 nmol/L) exacerbated the rate of cognitive decline to approximately 1-point per year (Morris et al., 2012). To date, no study has investigated the possible interaction of vitamin B₁₂ and folate status on telomere length.

B. Material and Methods

1. Study Population

Data from the National Health and Nutrition Examination Survey (NHANES) cycles 1999-2000 and 2001-2002 were used in our analyses because telomere length has only been measured in these two cycles. The NHANES is conducted by the National Center for Health Statistics (NCHS), which is part of the Centers for Disease Control and Prevention (CDC) and is responsible for publishing health statistics on a nationally representative sample of the US population. The NHANES survey data include information on demographic, socioeconomic, dietary, and health-related questions, as well as a laboratory and physical examination component. Participants are selected using a multistage cluster sampling design with oversampling occurring for persons aged 60 and older, African Americans, and Hispanics.

Eligible participants included men and women aged 20 years and older, who had blood stored for DNA analysis and subsequent mean T/S ratio data available. Of the original 7,827 participants with data on telomere length, 33 participants were excluded for missing data on the primary exposure variables (serum vitamin B₁₂ and methylmalonic acid (MMA), a biomarker of vitamin B₁₂ status). Serum vitamin B₁₂ and folate concentrations may appear artificially depressed or elevated in patients with chronic liver or kidney disease. Renal failure is a common reason for elevated serum B₁₂

levels (Patil, Bhosle, & Malik, 2016). Thus, we further excluded men and women with elevated serum creatinine (1.7 mg/dL (n = 81) and 1.5 mg/dl (n = 40), respectively), and those who answered yes to questions KIQ020 [KIQ022] (ever told you had weak/failing kidneys?) (n = 211), MCQ170L (do you still have a liver condition?) (n = 92), as well as those with elevated liver enzymes (ALT \geq 125 U/L, AST \geq 80 U/L, and Alkaline Phosphatase \geq 169 U/L; three standard deviations above the mean) (n = 66). Women who had a positive pregnancy test (code URXPREG) were also excluded from the study (n = 443), bringing the total loss of participants to 966. After reviewing the data for outliers and/or possible erroneous values via scatterplot data, participants with a telomere length greater than 10,000 base pairs (bp) (n=3), a serum vitamin B₁₂ level above 10,000 pmol/L (n=11), and/or a serum folate level 3 SD above the mean (\geq 93.3 nmol/L) (n = 108) were excluded from the sample population. The final sample size consisted of 6,739 men and women for whom data on telomere length, vitamin B₁₂ status, and folate status were available. The inclusion of all covariates (described below) in the adjusted multiple regression models brought the total number of participants for all analyses to 5,941.

2. Variables

Telomere length was our primary outcome. With each NHANES data collection cycle, blood samples are obtained and analyzed immediately. A small portion of the blood was reserved for future analysis of DNA, including telomere length. The telomere length assay was performed by Dr. Elizabeth Blackburn at the University of California, San Francisco (Cawthon, 2002). Polymerase chain reaction (PCR) methodology was used to measure the telomere length (T) relative to a standard reference DNA (S) producing a T/S ratio. Samples had been assayed in duplicate, three times on three different days,

resulting in 6 data points that were then averaged. The final interassay coefficient of variation was 6.5%. The formula reported in the NHANES database for converting T/S ratio to a more intuitive base pair variable is $(3,274 + 2,413 * (T/S))$. This formula is calculated based on the comparison of telomeric restriction fragment (TRF) length from Southern Blot analysis and T/S ratios using DNA samples from the human diploid fibroblast cell line IMR90 (Cawthon, 2002; Needham et al., 2013).

The main exposure variables were vitamin B₁₂ status, determined using both serum vitamin B₁₂ and methylmalonic acid (MMA) levels, and folate status. Vitamin B₁₂ and folate concentration measurements were performed using *Quantaphase II Folate/vitamin B₁₂* radioassay kit from Bio-Rad Laboratories. Analysis of MMA concentration was performed by gas chromatography and a mass selective detector using ion monitoring.

Based on a previous study examining vitamin B₁₂ status and macrocytic anemia (Morris et al., 2007), low vitamin B₁₂ status was defined as a serum vitamin B₁₂ level of ≤ 148 pmol/L and/or a serum MMA level of ≥ 210 nmol/L. This value is based on the upper end of the reference range for serum vitamin B₁₂-replete participants with normal creatinine levels. Folate status was categorized as suboptimal, sufficient, and elevated. Serum folate levels were based on guidelines set forth by the World Health Organization on serum and red blood cell folate concentrations for assessing folate status in populations; the suboptimal serum folate cut-off value was set at ≤ 13.4 nmol/L, and elevated serum folate at ≥ 45.3 nmol/L (World Health Organization, 2015). We defined sufficient folate status as those concentrations falling in between suboptimal and

elevated; thus, participants with serum folate levels greater than 13.4 nmol/L and less than 45.3 nmol/L were considered sufficient.

The demographic covariates for this study included age, sex and race/ethnicity. Age was a continuous variable in years, sex was coded “male” or “female,” and race/ethnicity was coded “White/non-Hispanic,” “Mexican/Hispanic,” “Black/non-Hispanic,” and “Other/Multi-Racial.”

Markers of socioeconomic status included educational status and annual household income. Educational status consisted of “ $\leq 11^{\text{th}}$ grade,” “high school graduate or GED equivalent,” and “some college or beyond.” Annual household income was determined to be non-significant in the model and was later dropped from further analyses.

Smoking status was categorized as “never,” “former,” and “current” smokers. Current smokers were further categorized by average number of cigarettes smoked per day. The “never” group consisted of those participants who answered no to question SMQ020 (smoked at least 100 cigarettes in life?). Former smokers were those who answered yes to SMQ020, but no to SMQ040 (do you now smoke cigarettes?). Current smokers were those who answered 1 or 2 (every day or some days, respectively) to question SMQ040 (Do you now smoke cigarettes?). This group was further categorized using the answer to question SMD070 (# cigarettes smoked/day now?), into <10 cigarettes/day, 10 to 19 cigarettes/day, and ≥ 20 cigarettes/day. The smoking variable was based on cigarette smoking only. Other methods of smoking or use of chewing tobacco were not assessed.

Alcohol intake was categorized as “never,” “former moderate,” “former heavy,” “current moderate,” and “current heavy.” There were 378 missing answers from respondents, six of whom answered “unknown” for intake on the questionnaire. Never drinkers were those answering no to having had at least 12 alcoholic drinks over the past 12 months and no to having had at least 12 alcoholic drinks in a lifetime. Former drinkers were those who answered yes to at least 12 drinks in a lifetime, but no to having drunk alcohol over the past 12 months. Former drinkers were divided into moderate and heavy intakes. Heavy drinkers answered yes to ever having \geq five drinks every day. Current drinkers were also divided into moderate and heavy alcohol intake. Current moderate drinkers consumed an average of \leq two drinks/day, while current heavy drinkers consumed \geq three drinks/day.

Blood glucose status was defined as normal, borderline, and diabetes using an algorithm of biochemical markers including percent hemoglobin A_{1c} (%A_{1c}), fasting and non-fasting plasma glucose levels, as well as medical history questionnaire data. Participants were categorized as having diabetes if they answered yes to taking insulin, to having been told they have diabetes by a doctor, and/or to taking oral glucose agents. Biochemical data for diabetes included a %A_{1c} of ≥ 6.5 , a fasting plasma glucose of ≥ 126 mg/dL, a plasma glucose of ≥ 200 mg/dL, and/or a fasting glucose of ≥ 126 mg/dL with an %A_{1c} ≥ 5.7 , but < 6.5 . Participants were categorized as borderline blood glucose status if they answered yes to having been told they have pre-diabetes. Biochemical data for borderline diabetes included a %A_{1c} ≥ 5.7 but $< 6.5\%$ with a fasting plasma glucose ≥ 100 mg/dL, but < 126 mg/dL. The remaining participants were categorized as having normal

blood glucose status if they did not meet the above criteria for impaired glucose. Those lacking data on two or more of these markers were categorized as missing.

3. Statistical Analysis

Data analyses were performed using STATA/IC version 13.1 to test our hypothesis that low vitamin B₁₂ status, as well as suboptimal and/or elevated folate status is associated with shorter telomere length in adult men and women. The continuous variables included age, body mass index (BMI), serum vitamin B₁₂ concentration, serum folate, methylmalonic acid level (MMA), C-reactive protein (CRP), and homocysteine levels, were reported as the unadjusted mean (\pm SD) by both vitamin B₁₂ and folate status. Categorical variables included sex, race, educational status, smoking status, alcohol intake status, and blood glucose status, and were reported as counts (%) by vitamin B₁₂ and folate status. The age-adjusted mean telomere length was calculated for the categorical variables by exposure status. Statistical analyses for vitamin B₁₂ status subgroups (low and sufficient) were performed using a two-sample T-test analyses. We used analysis of variance (ANOVA) and Chi-Squared test to examine whether covariate distributions differed across folate status subgroups (suboptimal, sufficient, and elevated). A *p*-value of < 0.05 was considered statistically significant for all tests. The main outcome and exposures had equal variances based on results of Levenes's test for homoscedasticity. ANOVA analysis was used to calculate the age-adjusted mean telomere length for vitamin B₁₂ and folate status subgroups, and to test for effect within our categorical covariates.

We tested our main hypotheses by evaluating vitamin B₁₂ and folate status and telomere length in a multiple linear regression model adjusting for potential confounding

variables. The above-mentioned covariates were assessed for their effect on telomere length or confounding with vitamin B₁₂ status and/or folate status. A multiple F-test was used to test the statistical significance of the parameters in the model. If a variable demonstrated no effect on the relationship between telomere length and our exposure variables ($p>0.05$) the parameter was eliminated from the model. The normality of the continuous variables was assessed via graphical methodology, C-reactive protein (CRP), body mass index (BMI) and plasma homocysteine were subsequently log-transformed to achieve greater homoscedasticity. We presented the telomere data as base pair units.

We fit linear regression models of vitamin B₁₂ status and folate status, on the outcome of telomere length adjusting for age alone, reported in *model 1*. Data are presented as the change in mean telomere length (bp). We used the log-transformed mean telomere (T/S) ratio in the linear regression analysis, prior to the calculation of bp, to determine the β coefficient for age. We later used this value in determining an estimate of biological aging associated with telomeric shortening. The regression coefficient for age was -0.0058 (0.6%), which corresponded to telomere length reduction of 14.1 bp per each one-year increase in participant age. *Model 2* represents the multiple linear regression that included sex and race, in addition to age. We chose to conduct the analyses in stages due to the particularly strong correlation of age, as well as sex and race, with telomere length. *Model 3* is the fully-adjusted model that included the above-mentioned covariates, as well as educational status, smoking status, alcohol intake status, BMI, blood glucose, serum homocysteine and CRP levels.

Given the importance of adequate vitamin B₁₂ status in folate metabolism, we created an interaction term of vitamin B₁₂ and folate status and formally tested it within

the three multiple linear regression models described above. Multiple F-test analyses were conducted to test for significance. We included age in the interaction term given the significant correlation between it and our outcome. We then formally tested the interaction term of B₁₂ status, folate status, and age for significance.

We repeated the model including the interaction term of B₁₂ and folate status with stratification on age to determine if there was effect modification by age and followed with multiple F-test analyses to test for significance. Analysis of variance (ANOVA) was used in determining age-adjusted mean telomere length for the subgroups of the interaction term and testing significance.

C. Results

1. Descriptive Data

Table 2.1 represents descriptive data for the 5,941 men and women in the study population (49.2% female). The mean age was 50.1 years (± 18.1). Non-Hispanic (NH) white participants made up 51.9% of the population, 17.4% were NH-black, 28.2% were Mexican/Hispanic, and 2.7% were multi-racial.

The mean telomere length for the total population was 5737.2 (7.4) base pairs (bp), and for males and females it was 5706.2 (10.4) and 5769.1 (10.5), respectively. NH-black participants had the longest mean telomere length (5844.8 bp [17.8]) of any racial/ethnic group, while Mexican/Hispanic participants had the shortest (5648.1 bp [13.9]).

The total population age-adjusted mean (SE) for serum vitamin B₁₂ and serum folate concentrations were 387.3 (3.8) pmol/L, and 32.0 (0.2) nmol/L, respectively. There

was an even distribution of B₁₂ and folate status between men and women. NH-white participants had the highest percentage of low B₁₂ status (19.1%), while NH-black participants had the lowest percentage (8.6%). A contrasting profile was found with folate status distribution, where NH-black participants had a higher percentage of suboptimal folate status (10.7%) compared to NH-white participants (5.3%). Elevated folate status was highest among NH-whites (23.0%) and lowest among NH-blacks (6.8%). Mexican/Hispanic participants fell between these percentages for both suboptimal and elevated folate status (6.5% and 10.2%, respectively). Approximately 2.2% of our study population had a mean serum vitamin B₁₂ level \leq 148 pmol/L, and 15.5% had low vitamin B₁₂ status. Approximately 6.6% of the study population was classified as suboptimal folate status, and 16.4% were classified as elevated folate status. Only 1.3% of the study population had low status in both nutrients, while 2.2% had low vitamin B₁₂ status coupled with elevated folate status. **Table 2.2** represents the age-adjusted mean telomere length (SE) for the interaction between vitamin B₁₂ and folate status.

a. Vitamin B₁₂ Status and Telomere Length

Table 2.3 presents data from multiple linear regression models of vitamin B₁₂ status, folate status, and telomere length. In the age-adjusted model (*Model 1*), low vitamin B₁₂ status was significantly associated with shorter telomere length ($\beta = -56.6$ bp, $p=0.006$). After adjusting for race and sex in addition to age (*Model 2*), the effect waned slightly while remaining significant ($\beta = -46.7$ bp, $p=0.025$). The fully-adjusted model included the addition of BMI, CRP and homocysteine levels, educational status, smoking

status, and alcohol intake (*Model 3*), low B₁₂ status remained significantly associated with shorter telomere length ($\beta = -55.1$ bp, $p=0.01$).

b. Folate Status and Telomere Length

In the age-adjusted linear regression model (*Model 1*), suboptimal folate status was negatively associated with mean telomere length ($\beta = -67.9$ bp, $p=0.023$) (**Table 2.3**). After adjusting for race and sex (*Model 2*), the association strengthened slightly, and suboptimal folate status continued to be associated with a shorter mean telomere length ($\beta = -76.1$ bp, $p=0.011$) compared to sufficient folate status. In the fully adjusted model for folate status (*Model 3*), suboptimal folate status remained significantly associated with a lower mean telomere length ($\beta = -68.9$ bp, $p=0.026$) compared to sufficient folate status. No association between elevated folate status and telomere length was found in any regression model.

c. Interaction of B₁₂ and Folate Status and Telomere Length

The interaction of B₁₂ status and folate status was evaluated in the fully adjusted model. The F-test yielded borderline significance ($p = 0.16$). When age was included in the interaction of B₁₂ and folate, the F-test was nearly significant at $p = 0.06$.

d. Interaction of B₁₂ and Folate Status and Telomere Length Stratified by Age

Table 2.4 represents data from the interaction of B₁₂ and folate status in the fully-adjusted multiple linear regression model, stratified by age categories of 20 – 39 years, 40 – 59 years, and >60 years. The F-tests testing the interaction between B₁₂ and folate

status, after stratifying by age, yielded p -values of 0.43, 0.02, and 0.23, respectively. There was a significant interaction between vitamin B₁₂, folate, and age within those 40 – 59 years, or middle-aged. Specifically, among middle-aged adults, low B₁₂/elevated folate status was significantly associated with a shorter mean telomere length ($\beta = -382.5$ bp, $p=0.007$) compared to those sufficient in both nutrients of the same age group. A significantly longer mean telomere length was observed in those with sufficient B₁₂ and elevated folate in the same age group ($\beta = 111.0$ bp, $p=0.009$). This association was not present in the other age groups. No interaction associations were found in those over 60 years of age.

D. Discussion

We observed an interaction among B₁₂ status, folate status and age and telomere length in adult men and women. Specifically, we found that low B₁₂ status in the presence of elevated folate level was significantly associated with a shorter mean telomere length in middle-aged adult men and women.

Few studies have investigated the role of one-carbon metabolism factors on telomere length. Of the studies conducted, none have reported an association with vitamin B₁₂ status and telomere length, while the relationship with folate status and telomere length has demonstrated varied results (Liu et al., 2013; Paul et al., 2009, 2014). To our knowledge, no studies have defined B₁₂ status utilizing serum vitamin B₁₂ along with MMA levels as we have, with the aim of investigating its association with telomere length in the presence of elevated folate.

Several studies have demonstrated negative health outcomes associated with elevated folate levels in the presence of low B₁₂ status (Brito et al., 2016; Deng, Wang, Wang, & Kwok, 2017; Morris et al., 2005; Morris et al., 2007, 2012; Selhub, Morris, & Jacques, 2007). We found that in middle-aged participants with low B₁₂ status, the presence of elevated folate levels may be detrimental to telomere length. Given the β coefficient for age in our simple linear regression model yielded an estimate of 14.1 bp telomeric attrition per each one-year increase in participant age, we used this coefficient to predict the amount of biological aging in those with low vitamin B₁₂ and elevated folate levels. The advanced telomeric attrition of 382 bp in this group can be interpreted as an additional 27 years of biological aging. In other words, among middle-aged adults, those with low vitamin B₁₂ status in the presence of elevated folate levels had biologically aged 27 years beyond their nutrient-sufficient, age-equivalent counterparts. While the mean age of the low B₁₂/elevated folate group is slightly higher than the total sample population mean age (54.0 years and 50.1 years, respectively), these middle-aged adults are biologically-aged closer to the oldest age category (mean age 72.0 years) with respect to telomere length.

Aging is the most influential, non-modifiable factor for telomere length. Youth is strongly associated with longer telomeres, and a linear decline occurs with age, regardless of intervening factors (Blackburn et al., 2015; Rode et al., 2015). Our data yielded less variation in telomere length among the younger (20–39 years) and older (>60 years) groups compared to those of middle age (40–59 years). The difference in mean telomere length (longest to shortest) within each of the three age categories (youngest to oldest) were 144.5 bp, 420.5 bp, and 143.3 bp, respectively. Thus, difference in mean telomere

length from the shortest to the longest, for those 40–59 years old is nearly three-fold that of the other two age groups, indicating greater telomeric variability within the middle-aged.

In contrast, when vitamin B₁₂ status was sufficient, elevated folate did not have a negative effect on telomere length in this same group of middle-aged adults and could be viewed as protective. Our findings were in line with those reported by Morris et al. (2007) who reported finding a protective effect of higher folate concentrations when vitamin B₁₂ levels were sufficient versus low. Morris et al. reported poorer cognitive functioning in older adults with low B₁₂ levels in the presence of elevated folate compared to normal folate levels. However, when B₁₂ was sufficient, less cognitive impairment was observed when folate levels were elevated compared to when they were normal (Morris et al., 2007).

The findings from our study are of public health importance, in that the consequences of low vitamin B₁₂ status may be more detrimental than previously thought. Historically, vitamin B₁₂ deficiency has often been masked by low folate concentrations; whereas, repleting serum folate levels corrects the overt symptomatology. However, left untreated, chronic vitamin B₁₂ deficiency will result in permanent neurological damage.

Our study's importance is two-fold. Foremost, to our knowledge, it is the first study to demonstrate that low vitamin B₁₂ status is associated with a significantly shorter mean telomere length in adult men and women. Previous studies neglected to use additional biomarkers, such as methylmalonic acid (MMA), as indicators of vitamin B₁₂ status, potentially misclassifying individuals with low B₁₂ as sufficient and biasing the

study toward the null (Liu et al., 2013; Paul et al., 2014). Telomeric shortening is a known response to oxidative stress, and shorter telomeres are associated with numerous chronic health conditions. If telomere length is used as an indicator of overall health status, individuals with low B₁₂ status are at greater risk than those sufficient in the nutrient.

Secondly, had our study solely used the current diagnostic cutoff for vitamin B₁₂ deficiency of <148 pmol/L to categorize status, we would have failed to identify 13.3% of the population who had low vitamin B₁₂ status. This would be equivalent to 43.2 million people in the United States, for whom low vitamin B₁₂ status could go undiagnosed. Low vitamin B₁₂ status could pose a significant medical and financial burden on our already strained healthcare system.

Our finding of a nearly significant interaction between B₁₂ and folate status, age and telomere length is of public health importance. We did find a significant association between low B₁₂ and elevated folate status and telomere length among middle-aged adults. The molecular mechanism behind the negative effects of elevated folate on telomere attrition in middle-aged adults with low B₁₂ status remains elusive. While our data indicate a possible protective effect provided by elevated folate when B₁₂ status is sufficient, elevated folate levels should be considered potentially harmful when vitamin B₁₂ status is low. Folic acid fortification programs may need to consider the possible population benefits of concomitant fortification with vitamin B₁₂. The potential negative impact of elevated folate status in the presence of low vitamin B₁₂ warrants further investigation.

E. Strengths and Limitations

This study provides a novel finding concerning vitamin B₁₂ status and telomere length. We utilized an additional measure of vitamin B₁₂ (MMA) to more accurately capture the B₁₂ status of our population. To our knowledge, this has not previously been done with the aim of investigating vitamin B₁₂ status and telomere length.

In addition, we investigated the joint classification of vitamin B₁₂ and folate status on telomere length, which we believe to be an innovative concept. Additionally, our data were taken from the NHANES (1999-2002), a nationally representative database, which provided a large and diverse sample population.

Limitations of this study include the cross-sectional study design structure, which does not allow for determinations of causality. Telomere length was measured using real-time PCR, which may not be as accurate at measuring absolute telomere length as the Southern Blot method. We also did not use weighted data; thus, we are unable to extrapolate percentage findings to the general U.S. population.

F. Areas of Further Study

While we did not investigate dietary or supplemental vitamin B₁₂ or folate/folic acid data in this study, we believe it is an area with future research potential for those studying food patterns, given the metabolic complexity and dichotomy of dietary sources for nutrients involved in one-carbon metabolism. Nonetheless, serum and plasma biomarkers are viewed as superior indicators of overall nutrient status to dietary intake data. In addition, we feel it would be of great interest to examine the association of vitamin B₆ (pyridoxal 5' phosphate, PLP) and telomere length given its role in one-carbon metabolism, as well as its role in neurotransmitter synthesis. To date, only one

study examined PLP in relation to telomere length and no association was found. However, the authors found a significant relationship between high plasma cysteine levels and shorter telomere length (Liu et al., 2013). Cysteine is produced from homocysteine in a double vitamin B₆-dependent enzymatic process with serine. The authors encourage caution in interpreting the results due to the lack of a significant linear trend.

CHAPTER 3

IRON STATUS AND TELOMERE LENGTH IN ADULT MEN AND WOMEN

A. Introduction

Telomeres are repetitive, non-coding nucleotide sequences at the end of chromosomes (5'- TTAGGG – 3') which protect them against damage during replication. Telomeric shortening occurs during cellular division, and cells undergo apoptosis when telomeres reach critical length, as part of the normal aging process. However, shorter telomeres have been associated with several chronic conditions such as cardiovascular disease, hypertension, type 2 diabetes, Alzheimer's disease, and certain cancers, independent of chronological age. Thus, suggesting its role as biomarker for aging in eukaryotic cells.

Oxidative stress has also been linked to several of the aforementioned chronic conditions, as well as the process of aging including shortened telomere length (Tomiyama et al., 2012; Von Zglinicki & Martin-Ruiz, 2005). Iron, an essential micronutrient, has been investigated for its role in oxidative damage. Nearly 65% of iron in the body is associated with hemoglobin; however, the importance of iron extends well beyond its oxygen carrying ability. The remaining iron is stored in hepatocytes as ferritin and hemosiderin (as Fe^{+3}), or in the spleen, bone marrow, and/or muscle tissue. Iron is involved in numerous metabolic processes as a part of heme in a cluster with sulfur, or by itself. It functions as a component of proteins involved in DNA synthesis, such as ribonucleotide reductase, and part of the catalytic site of cytochromes and peroxidases. Iron also acts as a cofactor for dozens of enzymes, including the reactive oxygen species (ROS) quenching enzymes catalase and myeloperoxidase (Gropper et al., 2009a).

Reactive oxygen species such as the superoxide radical (O_2^\bullet), hydroxyl radical ($^\bullet OH$), and hydrogen peroxide (H_2O_2) are generated in the body and are typically quenched by antioxidants and/or enzymes. However, a disruption in the equilibrium and subsequent ROS overload, can result in oxidative damage. Ferric iron (Fe^{+2}) is released in high concentrations from lysosomal apoptosis resulting from the cell damage. This excessive free iron can be a catalyst for further reactions (i.e. Fenton reaction) from which ROS are formed; thus perpetuating the cycle and leading to oxidative stress (Kepinska et al., 2015).

Research on elevated iron status and telomere length has demonstrated dichotomous results (Mainous III et al., 2014; Mainous III et al., 2013; Shin & Baik, 2017). Mainous III et al. found that high transferrin saturation percentage (TS%) was associated with shorter telomere length in their 2013 study of 1,009 predominantly Non-Hispanic white participants. However, in a subsequent study by Mainous III et al. (2014), the researchers found no significant association between elevated TS% and telomere length after adjusting for confounders in a sample of 669 participants of mixed race/ethnicity.

To our knowledge, no study has investigated the association of iron deficiency or iron deficiency anemia (IDA) on telomere length. Iron deficiency is a leading risk factor for death worldwide, and common among women and children (Zimmermann & Hurrell, 2007). In the United States, 9–11% of nonpregnant women between the ages of 16 and 49 years are iron deficient, with 2–5% having IDA (World Health Organization, 2017). Among older adults, IDA is associated with a higher risk of falls, increased hospitalization, impaired mental function, and an increased risk of death (World Health

Organization, 2017). Oxidative stress and lower antioxidant levels have been reported in iron deficient subjects (Pra et al., 2012). Iron restriction has been shown to decrease DNA synthesis and cell proliferation, and reduce ribonucleotide reductase activity, all leading to genomic instability (Pra et al., 2012). Thus, providing more reason to explore the association of ID/IDA on telomere length.

Our aim was to investigate the association of iron status on telomere length in adult men and women from a nationally representative sample. We hypothesized that men and women with either suboptimal or elevated iron status would have a shorter mean telomere length compared to those with normal iron status.

B. Material and Methods

1. Study Population

Data from NHANES (National Health and Nutrition Examination Survey) cycles 1999-2000 and 2001-2002 were used in the analyses. The NHANES is conducted by the National Center for Health Statistics (NCHS), which is part of the Centers for Disease Control and Prevention (CDC) and is responsible for publishing health statistics on a nationally representative sample of the US population. The NHANES survey data include information on demographic, socioeconomic, dietary, and health-related questions, as well as a laboratory and physical examination component. Participants are selected using a multistage cluster sampling design with oversampling of persons aged 60 and older, African Americans, and Hispanics.

Eligible participants included men and women aged 20 years and older who had blood stored for DNA analysis and subsequent mean T/S ratio data available (n = 7827). All eligible participants had complete data on sex, C-reactive protein (CRP) levels, and at

least two of the following variables used to categorize iron status: serum iron, serum ferritin, erythrocyte protoporphyrin concentration, transferrin saturation percentage, and/or hemoglobin levels.

Exclusion criteria included men and women with an elevated serum creatinine (1.7 mg/dL (n = 85) or 1.5 mg/dl (n = 45), respectively), and those with liver and kidney disease. The kidneys are responsible for producing erythropoietin, a hormone that prompts bone marrow to synthesize red blood cells (RBC). Thus, kidney disease often results in fewer RBCs and anemia; while chronic liver disease is frequently associated with hematological abnormalities including iron deficiency anemia and iron overload.

Liver disease was defined as those who answered yes MCQ170L (do you still have a liver condition) (n = 155), as well as those with elevated liver enzymes (ALT \geq 125 U/L, AST \geq 80 U/L, and Alkaline Phosphatase \geq 169 U/L; three standard deviations above the mean) (n = 2). Kidney disease was defined as those who answered yes to questions KIQ020 (ever told you had weak/failing kidneys) (n = 211). Women with a positive pregnancy test (code URXPREG) were also excluded (n = 445), bringing the loss of participants to 943. After reviewing for outliers and/or possible erroneous values via scatterplot data, participants with serum ferritin levels $>$ 500 ng/mL (n=172), mean telomere length greater than 10,000 bp (n=3), and those with a serum iron level greater than three standard deviations above the mean were excluded from the study (n=10). The inclusion of covariates (described below) brought the total number of participants with complete data available for the multiple linear regression analyses to 5,908.

2. Variables

a. Telomere Length

Telomere length was our primary outcome. With each NHANES data collection cycle, blood samples are obtained and analyzed immediately. A small portion of the blood was reserved for future analysis of DNA, including telomere length. The telomere length assay was performed by Dr. Elizabeth Blackburn at the University of California, San Francisco (Cawthon, 2002). Polymerase chain reaction (PCR) methodology was used to measure the telomere length (T) relative to a standard reference DNA (S) producing a T/S ratio. Samples had been assayed in duplicate, three times on three different days, resulting in 6 data points that were then averaged. The final interassay coefficient of variation was 6.5%. The formula reported in the NHANES database for converting T/S ratio to a more intuitive base pair variable is $(3,274 + 2,413 * (T/S))$. This formula is calculated based on the comparison of telomeric restriction fragment (TRF) length from Southern Blot analysis and T/S ratios using DNA samples from the human diploid fibroblast cell line IMR90 (Cawthon, 2002; Needham et al., 2013).

b. Iron Status

The main exposure was iron status, which was determined using an algorithm of serum iron (Fe), serum ferritin, erythrocyte protoporphyrin level (EPP), transferrin saturation percentage (TS%), C-reactive protein level (CRP), hemoglobin level (hb) and sex. Cutoff levels for determining iron status were taken from the World Health Organization (WHO) (World Health Organization, 2001) and Iron Status Indicators (Cusick, Looker, Cogswell, Pfeiffer, & Grummer-Strawn, 2008).

Serum iron measures the amount of ferric (Fe^{3+}) iron in the body mainly bound to transferrin (a primary iron transport protein). This measurement does not capture the divalent iron bound to hemoglobin. Serum iron level alone is not a good indicator of

overall iron stores, nor is it a sensitive measure for iron deficiency (Cusick et al., 2008). Therefore, it was used in conjunction with other measures of iron in defining iron status.

Ferritin, the primary storage protein for iron, is widely used to indicate available iron in the body. Low ferritin levels (<15 ug/L) are highly associated with iron deficiency (ID) and iron deficiency anemia (IDA) (Cusick et al., 2008; World Health Organization, 2001). However, ferritin is an acute phase respondent and levels will rise in response to inflammation. In participants with elevated ferritin levels (>200 ng/mL and >300 ng/mL for women and men, respectively), we used a CRP cutoff value of >10 mg/L to indicate a possible acute-phase response of ferritin to inflammation. These participants were classified with sufficient iron status if they did not have at least one additional biomarker of abnormal iron status.

Hemoglobin (Hb) is also widely used in diagnosing anemia, specifically IDA, since it binds approximately 70% of the body's total iron. We used the WHO cutoff for IDA of <12 g/dL Hb for women, and <13 g/dL Hb for men as an acceptable co-biomarker for suboptimal iron status (World Health Organization, 2001).

Erythrocyte protoporphyrin (EPP) is a precursor of heme and levels rise when iron stores are inadequate for hemoglobin production. In addition to ID, infection, inflammation, and lead-poisoning will also result in an elevated EPP level. In the absence of infection, measurement of EPP is the preferred method for detecting ID once ferritin levels drop below normal (World Health Organization, 2001).

Transferrin saturation percentage (TS%) is the measure of total iron bound to transferrin, an iron transport protein, and is the ratio of serum iron to the total iron binding capacity of transferrin (TIBC). A TS% <16 is indicative of IDA (Zimmermann &

Hurrell, 2007), while excess of $\geq 55\%$ indicates iron overload, or hemochromatosis (Mainous III, Gill, & Carek, 2004). A TS% of < 20 is considered indicative of suboptimal iron status.

3. Iron Status Categorization

We used the above-mentioned biomarkers of iron status to categorize participants as having suboptimal, sufficient, or elevated iron status. Participants were considered to have suboptimal iron status if they had two or more of the following criteria: a serum ferritin level $\leq 15 \mu\text{g/L}$, EPP level $> 80 \mu\text{g/dL}$, TS% < 20 , or Hb < 12 for women and < 13 for men.

To define elevated iron status, we used one or more of the following criteria: TS% ≥ 50 , serum iron level of $\geq 155 \mu\text{g/dL}$ for women or $\geq 160 \mu\text{g/dL}$ for men, serum ferritin level of $\geq 150 \mu\text{g/L}$ for women or $\geq 200 \mu\text{g/L}$ for men along with a CRP $< 10 \text{ mg/L}$. Participants with elevated ferritin and elevated CRP levels, but no other abnormal markers for iron status were classified as sufficient. Participants were classified with sufficient iron status if they did not meet the set criteria for suboptimal or elevated iron status. Thus, participants sufficient in iron had serum ferritin levels $\geq 15 \mu\text{g/L}$ and $< 150 \mu\text{g/L}$ for women and $< 200 \mu\text{g/L}$ for men, EPP levels $< 80 \mu\text{g/dL}$, Hb $\geq 12 \text{ g/dL}$ for women and $\geq 13 \text{ g/dL}$ for men, TS% ≥ 20 and < 50 , and serum iron levels $< 155 \mu\text{g/dL}$ for women and $< 160 \mu\text{g/dL}$ for men.

4. Demographic Data

The demographic covariates for this study included age, sex and race/ethnicity. Age was a continuous variable in years, sex was coded “male” or “female,” and

race/ethnicity was coded “White/non-Hispanic,” “Mexican/Hispanic,” “Black/non-Hispanic,” and “Other/Multi-Racial.”

Markers of socioeconomic status included educational status and annual household income. Educational status consisted of “ $\leq 11^{\text{th}}$ grade,” “high school graduate or GED equivalent,” and “some college or beyond.” Annual household income was determined to be non-significant in the model and was later dropped from further analyses.

Smoking status was categorized as “never,” “former,” and “current” smokers. Current smokers were further categorized by average number of cigarettes smoked per day. The “never” group consisted of those participants who answered no to question SMQ020 (smoked at least 100 cigarettes in life?). Former smokers were those who answered yes to SMQ020, but no to SMQ040 (do you now smoke cigarettes?). Current smokers were those who answered 1 or 2 (every day or some days, respectively) to question SMQ040 (do you now smoke cigarettes?). This group was further categorized using the answer to question SMD070 (# cigarettes smoked/day now?), into < 10 cigarettes/day, 10 to 19 cigarettes/day, and ≥ 20 cigarettes/day. The smoking variable was based on cigarette smoking only. Other methods of smoking or use of chewing tobacco were not assessed.

Alcohol intake was categorized as “never,” “former moderate,” “former heavy,” “current moderate,” and “current heavy.” Never drinkers were those answering no to having had at least 12 alcoholic drinks over the past 12 months and no to having had at least 12 alcoholic drinks in a lifetime. Former drinkers were those who answered yes to at least 12 drinks in a lifetime, but no to having consumed alcohol over the past 12 months.

Former drinkers were divided into moderate and heavy intakes. Heavy drinkers answered yes to ever having \geq five drinks every day. Current drinkers were also divided into moderate and heavy alcohol intake. Current moderate drinkers consumed an average of \leq two drinks/day, while current heavy drinkers consumed \geq three drinks/day. Blood glucose status was defined as normal, borderline, and diabetes using an algorithm of biochemical markers including percent hemoglobin A_{1c} (%A_{1c}), fasting and non-fasting plasma glucose levels, as well as medical history questionnaire data. Participants were categorized as having diabetes if they answered yes to taking insulin, to having been told they have diabetes by a doctor, and/or to taking oral glucose agents. Biochemical data for diabetes included a %A_{1c} of ≥ 6.5 , a fasting plasma glucose of ≥ 126 mg/dL, a plasma glucose of ≥ 200 mg/dL, and/or a fasting glucose of ≥ 126 mg/dL with an %A_{1c} ≥ 5.7 , but < 6.5 . Participants were categorized as borderline blood glucose status if they answered yes to having been told they have pre-diabetes. Biochemical data for borderline diabetes included a %A_{1c} ≥ 5.7 but $< 6.5\%$ with a fasting plasma glucose ≥ 100 mg/dL, but < 126 mg/dL. The remaining participants were categorized as having normal blood glucose status if they did not meet the above criteria for impaired glucose. Those lacking data on two or more of these markers were categorized as missing.

C. Statistical Analysis

Data analyses were performed using STATA/IC version 13.1 to test our hypothesis that low, as well as elevated iron status is associated with shorter telomere length in adult men and women. The continuous variables included age, body mass index (BMI), serum iron concentration, serum ferritin level, transferrin saturation percentage

(TS%), erythrocyte protoporphyrin (EPP) concentration, and hemoglobin (Hb) level were reported as the adjusted mean (SE) by sex and iron status (suboptimal, sufficient, elevated). Categorical variables included race, educational status, annual household income, smoking status, alcohol intake status, and blood glucose status, and were reported as counts (%) by sex and iron status. The age-adjusted mean telomere length was calculated for the categorical variables by sex and exposure status. Two-sample T-test, Analysis of variance (ANOVA) and Chi-Squared test were used to examine whether covariate distributions differed across sex and iron status subgroups (suboptimal, sufficient, elevated). A p -value of < 0.05 was considered statistically significant for all tests. The main outcome and exposures had equal variances based on results of Levenes's test for homoscedasticity.

We tested our main hypotheses by iron status and telomere length in a multiple linear regression model adjusting for potential confounding variables. The above-mentioned covariates were assessed for their effect on telomere length or confounding with iron status. A multiple F-test was used to test the significance of the parameters in the model. If a variable demonstrated no effect on the relationship between telomere length and our exposure variables ($p > 0.05$) the parameter was eliminated from the model. The normality of the continuous variables was assessed via graphical methodology, T/S ratio and BMI were subsequently log-transformed to achieve greater homoscedasticity. We presented the telomere data as base pair units.

We conducted a linear regression model of iron status and telomere length adjusting for age alone, reported in *model 1*. Data are presented as the change in mean telomere length base pair (bp). We used the log-transformed mean telomere (T/S) ratio in

the linear regression analysis, prior to the calculation of bp, to determine the β coefficient for age. We later used this value in determining an estimate of telomeric shortening associated with biological aging. The regression coefficient for age was -0.0059 (0.6%), which corresponded to telomere length reduction of 14.3 bp per each one-year increase in participant age. *Model 2* represents the multiple linear regression that included race and sex, in addition to age. We chose to conduct the analyses in stages due to the particularly strong correlation of these three variables with telomere length. *Model 3* is the fully-adjusted model that builds on *model 2* by including BMI, educational status, smoking status, and alcohol intake status.

To investigate effect modification, we created interaction terms for iron status and sex, race, and age. We tested the interaction terms for significance in the various models. If significant, we stratified the linear regression models by the variable and utilized multiple F-test analysis for significance.

D. Results

1. Descriptive Statistics

Table 3.1 presents descriptive data for the 5,908 participants by sex in our study population (50.4% female). The mean ages for men and women were 50.0 and 50.4 years, respectively. Non-Hispanic (NH) white participants made up 52.4% of the population, 16.9% were NH-black, 28.1% were Mexican/Hispanic, and 2.6% were multi-racial. Given the small sample size of the multi-racial group, we consider any significant findings to be only suggestive.

The mean age of men with suboptimal iron status was significantly older than the mean age of women with suboptimal iron status. Whereas the mean age for women with elevated iron status was significantly higher compared to men ($p < 0.001$). Furthermore, men and women with suboptimal and elevated iron status, separately, differed significantly in all iron status parameters except for erythrocyte protoporphyrin levels among those with suboptimal iron status and serum iron among those with elevated iron status. C-reactive protein (CRP) was significantly higher among those with suboptimal iron status compared to those with sufficient iron status, and tended to be higher overall, in women compared to men. Body mass index (BMI) also tended to be higher in women compared to men (**Table 3.1**).

A greater percentage of women (17.3%) had suboptimal iron status compared to men (4.0%). Whereas men had a higher prevalence of elevated iron status compared to women (30.5% and 13.3%, respectively). Elevated iron status was similar among all racial/ethnic groups (**Table 3.1**). NH-black participants were more likely to have suboptimal iron status (14.6%) compared to NH-white (8.8%), Mexican/Hispanic (12.2%), or multi-racial (7.7%) participants. When stratified by sex, a significantly higher percentage of NH-black and Mexican/Hispanic women had suboptimal iron status compared to NH-white women.

The distribution of blood glucose status was similar between men and women, with 11.4% of men and 10.5% of women having diabetes. Among male participants with diabetes, 39.3% had elevated iron status, while only 29.4% and 28.9% of men with normal and borderline blood glucose, respectively, had elevated iron status. A similar pattern was found in women; 18.5% of women with diabetes had elevated iron status,

whereas 12.2% and 15.7% of those with normal and borderline blood glucose, respectively, had elevated iron status (**Table 3.1**).

Women of all ethnic/racial groups had longer mean telomere lengths than men; furthermore, NH-black women had the longest age-adjusted mean telomere length (5943.5 bp) of all ethnic/racial groups, followed by NH-black men (5855.5 bp) (**Table 3.2**).

2. Inferential Statistics

a. Iron Status and Telomere Length

Table 3.3 represents data from multiple linear regression models of iron status and telomere length. In the age-adjusted model (*Model 1*), elevated iron status was significantly associated with shorter telomere length ($\beta = -48.8$ bp, $p=0.007$). After adjusting for race and sex in addition to age (*Model 2*), the effect was reduced slightly while remaining significant ($\beta = -41.1$ bp, $p=0.025$). The fully-adjusted model included the addition of body mass index (BMI), educational status, smoking status, and alcohol intake status (*Model 3*). Annual household income and blood glucose levels were not significant in the model and were therefore omitted from further investigation. In the fully adjusted model (*Model 3*), elevated iron status remained significantly associated with shorter telomere length ($\beta = -36.7$ bp, $p=0.045$). Suboptimal iron status was not associated with telomere length in any models analyzed.

The model-based estimate of age-related telomeric attrition in this study was -0.0059 (0.6%), which corresponded to 14.3 bp. Thus, each year of chronological “normal” aging was associated with a telomeric loss of 14.3 bp. The difference in

telomere length in participants with elevated iron status (-36.7 bp) would be equivalent to 2.6 years of additional “biological” aging in this group.

b. Interaction of Iron Status and Sex, Race, or Age

The interaction term of iron status and sex was not significant ($p = 0.67$), nor were the interaction terms of iron status and race ($p = 0.61$) or iron status and age ($p = 0.63$). Thus, no further analyses were performed on the interaction terms.

E. Discussion

There is evidence that elevated iron status is associated with shorter telomere length (Mainous III et al., 2013), but the results have been inconsistent (Mainous III et al., 2014). We expanded our investigation to include suboptimal iron status, in addition to elevated iron status, given the high prevalence of iron deficiency (ID) in the United States, where 10% of Non-Hispanic (NH)-whites, 19% of NH-blacks and 22% of Mexican/Hispanics aged 20 – 49 years are iron deficient. (US Department of Health and Human Services. Centers for Disease Control and Prevention., 2002).

We found a small, but statistically significant association between elevated iron status and shorter mean telomere length among adult men and women from this nationally representative sample. The model-based estimate for biological aging using the T/S ratio β -coefficient for age of -0.006% (equivalent to -14.3 base pairs) suggested that those with elevated iron status are biologically two and one-half years older than their iron-sufficient counterparts. Although the distribution of iron data was different between men and women, as well as between the ethnic/racial groups and age, we did not find a

significant interaction between the tested variables, indicating no effect modification by sex, race, or age on iron status.

Elevated iron status, primarily as hemochromatosis, has been associated with detrimental health outcomes including cirrhosis of the liver, pancreatic dysfunction leading to diabetes, and cardiovascular disease (Richardson, McNamee, & Simmonds, 2018). We were not able to assess occurrence of the *HFE* mutation which leads to hereditary hemochromatosis in our study population. However, Mainous III and colleagues (2013) found that elevated iron phenotype, but not hemochromatosis (*HFE* genotype) was associated with shorter telomere length, indicating that it may be the elevated iron status rather than the specific genetic makeup that is associated with telomeric attrition.

Although we did not find a significant association with impaired blood glucose levels and telomere length, we did note that a significantly greater percentage of participants with diabetes had an elevated iron status (39.3%) compared to those with normal blood glucose levels (29.4%). This finding is in concordance with previous data supporting an increased risk of diabetes associated with elevated iron status (Montonen et al., 2012).

Non-Hispanic (NH) black participants were more likely to have suboptimal iron status, as were women; however, this did not reflect in a shorter mean telomere length for either group. Overall, we did not find a significant association between suboptimal iron status and shorter telomere length, although our study had sufficient power to observe a relationship if one indeed existed. While all participants were categorized with at least two biomarkers of iron deficiency, we used a transferrin saturation percentage (TS%)

cutoff of <20%, rather than the 16% indicated by the World Health Organization (WHO) to define iron deficiency (ID). Nonetheless, the cutoff of <20% had also been suggested in defining poor iron status (Daniels, 2010). In our study, 195 of the 750 participants with suboptimal iron status had a TS% ≥ 16 but <20%. Of these participants, 112 had serum ferritin levels >15 $\mu\text{g/L}$ but met at least one of the remaining criteria for suboptimal iron status. No difference was found when suboptimal iron status was classified using TS% of ≤ 16 compared to <20% in our analyses.

Iron deficiency is the most common nutritional disorder in the world, and is associated with increased levels of oxidative stress and decreased antioxidant defenses (Pra et al., 2012). While iron is essential for DNA synthesis via ribonucleotide reductase, suboptimal iron status does not appear to be associated with telomeric attrition as we initially hypothesized. To our knowledge, there are no published studies on iron deficiency and telomere length.

F. Strengths and Limitations

This study filled a knowledge gap on suboptimal iron status and telomere length in adult men and women. We utilized multiple biomarkers of iron in determining suboptimal and elevated iron status. By doing so, we strengthened our ability to correctly identify participants who were truly suboptimal or who had elevated iron status. Furthermore, our data was taken from the National Health and Nutrition Examination Survey (NHANES 1999 – 2002) which provided a large, diverse sample population.

1. Limitations

Micronutrient status can be difficult to study in *vivo* due to the challenges in obtaining valid biomarker data. Determinants of iron status are known to fluctuate depending on certain health parameters. For example, chronic disease and acute infections may result in abnormal hematology; thus, making an accurate assessment based on one timepoint come into question. It is possible, despite our best efforts, that we misclassified participants within the suboptimal and sufficient groups, thus nullifying the results. Further limitations include the cross-sectional study design structure, which does not allow for determinations of causality. We also did not use weighted data; thus, we are unable to extrapolate percentage findings to the general U.S. population.

G. Area of Future Research

Further research on the potential association between suboptimal iron status and telomere length is warranted. Well-designed prospective studies that include dietary and biomarker data on micronutrient intake and status would contribute to answering questions on causality.

H. Summary

A common thread in the progression of aging is the accrual of genetic/DNA damage. Premature aging diseases, such as Werner and Bloom syndromes, have provided insight into how DNA instability accelerates the aging process, but do not completely elucidate the matter. Telomeric attrition is considered to be a primary hallmark of aging and thus, measurement of telomere length has been proposed to be an indicator of

biological aging (Aviv, 2002; López-Otín et al., 2013). Nutrient status is a modifiable factor that has been associated with telomere length.

In our study, less than optimal micronutrient status was significantly associated with a shorter mean telomere length. Specifically, after stratifying by age, we found a significant interaction between low vitamin B₁₂ and elevated folate status in middle-aged adults. The telomeric attrition of 382 base pairs in this group was equivalent to nearly 27 years of additional biological aging using the model-based estimate for aging. This interaction was not observed in the younger or older age groups; however, middle-age could be considered the most vulnerable time for telomeres. Telomere length in this age group was also the most variable in our study, suggesting that telomeres during middle-age may be more susceptible to factors influencing attrition, such as poor nutrient status.

Similarly, we found a negative association between elevated iron status and telomere length. Although we hypothesized a relationship, we did not find an association between suboptimal iron status and telomere length. Furthermore, we did not find an interaction between iron and age, and therefore could not support our previous findings in middle-aged adults. Overall, our study was novel in approach, and successfully contributed to the current literature on micronutrient status and telomere length.

APPENDIX
TABLES

Table 1.1:

Age-adjusted characteristics of eligible participants from the National Health and Nutrition Examination Survey (1999-2002) by vitamin B₁₂ and folate status (N=5,941).

	Total Mean (SE)	Low B ₁₂ status ²	Sufficient B ₁₂ Status	<i>p</i> -values ⁵	Suboptimal Folate Status ³	Sufficient Folate Status	Elevated Folate Status ⁴	<i>p</i> -values ⁵
Age (yrs)	50.1 (0.2)	58.0 (0.6)	48.7 (0.3)	< 0.001	43.7 (0.9)	48.5 (0.3)	60.5 (0.6)	< 0.001
Body Mass Index (kg/m ²)	28.3 (0.1)	27.9 (0.2)	28.4 (0.1)	<0.001	29.0 (0.3)	28.4 (0.1)	27.3 (0.2)	< 0.001
Serum B12 (pmol/L)	387.3 (3.8)	291.0 (9.7)	405.0 (4.0)	< 0.001	325.8 (14.6)	374.3 (4.2)	473.3 (9.5)	< 0.001
MMA (nmol/L)	151.6 (1.3)	305.2 (2.9)	123.3 (1.2)	< 0.001	167.3 (5.4)	151.6 (1.5)	131.0 (3.5)	NS
Serum Folate (nmol/L)	32.0 (0.2)	28.3 (0.5)	32.7 (0.2)	0.02	11.3 (0.4)	27.9 (0.1)	59.5 (0.3)	< 0.001
CRP (mg/L)	4.5 (0.1)	4.4 (0.3)	4.5 (0.1)	NS	6.2 (0.4)	4.4 (0.1)	4.0 (0.3)	0.005
Homocysteine (umol/L)	8.7 (0.0)	10.5 (0.1)	8.4 (0.0)	< 0.001	12.3 (0.2)	8.7 (0.0)	7.4 (0.1)	< 0.001
	Total N (%)	Low B ₁₂ Status ²	Sufficient B ₁₂ Status	<i>p</i> -values ⁵	Suboptimal Folate Status ³	Sufficient Folate Status	Elevated Folate Status ⁴	<i>p</i> -values ⁵
Gender								
Males	3016 (50.8)	488 (53.0)	2528 (50.4)	NS	215 (54.7)	2408 (52.7)	393 (40.3)	< 0.001
Female	2925 (49.2)	433 (47.0)	2492 (49.6)		178 (45.3)	2165 (47.3)	582 (59.7)	
Race								
Non-Hispanic White	3085 (51.9)	589 (64.0)	2496 (49.7)	< 0.001	164 (41.7)	2212 (48.4)	709 (72.7)	< 0.001
Mexican/Hispanic	1676 (28.2)	210 (22.8)	1466 (29.2)		109 (27.7)	1396 (30.5)	171 (17.5)	
Non-Hispanic Black	1021 (17.4)	88 (9.6)	933 (18.6)		109 (27.7)	843 (18.4)	69 (7.1)	
Multi-Racial	159 (2.7)	34 (3.7)	125 (2.5)		11 (2.8)	122 (2.7)	26 (2.7)	
Education								
Less than 11 th Grade	1931 (32.5)	354 (38.4)	1577 (31.4)	< 0.001	93 (23.7)	1519 (33.2)	255 (26.2)	< 0.001
High School Graduate	1410 (23.7)	243 (26.4)	1167 (23.3)		157 (40.0)	1106 (24.2)	211 (21.6)	
Some College/Beyond	2600 (43.8)	324 (35.2)	2276 (45.3)		143 (36.4)	1948 (42.6)	509 (52.2)	
Income								
Less than \$19K/yr	1222 (23.0)	250 (30.0)	972 (21.7)	< 0.001	101 (29.3)	925 (22.6)	196 (22.4)	0.01
\$20K - \$54K/yr	2298 (43.3)	351 (42.1)	1947 (43.5)		152 (44.1)	1775 (43.4)	371 (42.3)	
Greater than \$55K/yr	1789 (33.7)	233 (27.9)	1556 (34.8)		92 (26.7)	1388 (34.0)	309 (35.3)	
Smoking Status								
Never Smoker	3139 (52.8)	461 (50.1)	2678 (53.4)	0.026	176 (44.8)	2403 (52.6)	560 (57.4)	< 0.001
Former Smoker	1650 (27.8)	286 (31.1)	1364 (27.2)		63 (16.0)	1269 (27.8)	318 (32.6)	
<10 cigs/d	274 (4.6)	33 (3.6)	241 (4.8)		34 (8.7)	220 (4.8)	20 (2.1)	
10 – 19 cigs/d	338 (5.7)	46 (5.0)	292 (5.8)		42 (10.7)	276 (6.0)	20 (2.1)	
>=20 cigs/d	540 (9.0)	95 (10.3)	445 (8.9)		78 (19.9)	405 (8.9)	57 (5.9)	
Alcohol Intake								
Never	888 (15.0)	181 (19.7)	707 (14.1)	< 0.001	47 (12.0)	672 (14.7)	169 (17.3)	< 0.001
Former Moderate	866 (14.6)	155 (16.8)	711 (14.2)		46 (11.7)	644 (14.1)	176 (18.1)	
Former Heavy	266 (4.5)	44 (4.8)	222 (4.4)		20 (5.1)	211 (4.6)	35 (3.6)	
Current Moderate	2516 (42.4)	359 (39.0)	2157 (43.0)		139 (35.4)	1903 (41.6)	474 (48.6)	

Current Heavy	1405 (23.7)	182 (19.8)	1223 (24.4)		141 (35.9)	1143 (25.0)	121 (12.4)	
Blood Glucose Status	4452 (75.0)	634 (68.8)	3818 (76.1)	<0.001	309 (78.6)	3439 (75.2)	704 (72.2)	0.043
Normal	816 (13.7)	147 (16.0)	669 (13.3)		54 (13.8)	612 (13.4)	150 (15.4)	
Borderline	673 (11.3)	140 (15.2)	533 (10.6)		30 (7.6)	522 (11.4)	121 (12.4)	
Diabetes								

Continuous data presented as age-adjusted mean (SE)

Categorical data presented as N (%) by B₁₂ status and folate status

¹ Low vitamin B12 status defined as serum B12 <148 pmol/L and/or MMA ≥210 nmol/L

² Two-sample t-test, significance set at <0.05

³ Suboptimal folate defined as serum folate ≤13.4 nmol/L

⁴ Elevated folate status defined as serum folate ≥45.3 nmol/L

⁵ Chi-Squared test, significance set at <0.05

Table 1.2:

Mean telomere length of eligible participants from the National Health and Nutrition Examination Survey (1999-2002) by joint classification of vitamin B₁₂ and folate status adjusted for age (N=5,941).¹

	Total	Sufficient B ₁₂ Sufficient Folate (N = 4,366)	Sufficient B ₁₂ Suboptimal Folate ³ (N = 366)	Sufficient B ₁₂ Elevated Folate ⁴ (N = 922)	Low B ₁₂ ² Sufficient Folate (N = 838)	Low B ₁₂ ² Suboptimal Folate ³ (N = 96)	Low B ₁₂ ² Elevated Folate ⁴ (N = 151)	<i>p</i> -values
RTL (base pairs)	5749.2 (7.0)	5748.4 (9.3)	5661.5 (32.4)	5773.8 (20.0)	5681.5 (21.8)	5703.0 (64.6)	5674.9 (50.9)	< 0.001

¹ Adjusted for age, presented as mean (SE)

² Low vitamin B₁₂ status defined as serum B₁₂ <148 pmol/L and/or MMA ≥210 nmol/L

³ Suboptimal folate defined as serum folate ≤13.4 nmol/L

⁴ Elevated folate status defined as serum folate ≥45.3 nmol/L

⁵ ANOVA, Significance set at <0.05

Table 1.3:

Multiple linear regression models of vitamin B₁₂ status, folate status, and telomere length in participants from the National Health and Nutrition Examination Survey (1999-2002) N=5,941.

	Model 1 ¹			Model 2 ²			Model 3 ³		
	β, (95%CI)	p-value	R ²	β, (95%CI)	p-value	R ²	β, (95%CI)	p-value	R ²
B₁₂ Status⁴									
Low	-56.6 (-97.2, -15.9)	0.006	0.169	-46.7 (-87.4, -6.0)	0.025	0.184	-55.1 (-97.1, -13.1)	0.01	0.193
Sufficient	Ref	---		Ref	---		Ref	---	
Folate Status⁵									
Suboptimal	-67.9 (-126.6, -9.2)	0.023	0.169	-76.1 (-134.5, -17.8)	0.011	0.184	-68.9 (-129.4, -8.4)	0.026	0.193
Sufficient	Ref	---		Ref	---		Ref	---	
Elevated	25.6 (-14.9, 66.1)	0.216		14.0 (-27.0, 55.0)	0.504		7.0 (-34.6, 48.5)	0.742	

¹ Adjusted for age

² Adjusted for age, sex, race, B₁₂ or folate status

³ Adjusted for age, sex, race, B₁₂ or folate status body mass index[†], C-reactive protein level[†], homocysteine level[†], educational status, smoking status, alcohol intake status

⁴ Low B₁₂ status defined as serum B₁₂ ≤148 pmol/L and/or MMA ≥210 nmol/L

⁵ Suboptimal folate status defined as serum folate ≤13.4 nmol/L; elevated folate status defined as serum folate ≥45.3 nmol/L

Table 1.4:

Telomere length by joint classification of vitamin B₁₂ and folate status stratified by age in eligible participants from the National Health and Nutrition Examination Survey (1999-2002) (N=5,941).¹

Mean Telomere Length (bp)						
B ₁₂ /Folate Status	20 – 39 years (n = 2068)	<i>p</i>	40 – 59 years (n = 1935)	<i>p</i>	>60 years (n = 1938)	<i>p</i>
Sufficient B ₁₂ /Sufficient Folate	Ref	--	Ref	--	Ref	--
Sufficient B ₁₂ /Suboptimal Folate ³	-108.1 (-214.2, -1.9)	0.046	-108.3 (-223.6, 7.0)	0.07	-32.0 (-180.9, 116.9)	0.673
Sufficient B ₁₂ /Elevated Folate ⁴	-25.6 (-128.8, 77.7)	0.627	111.0 (28.2, 193.9)	0.009	-33.7 (-92.2, 24.8)	0.259
Low B ₁₂ ² /Sufficient Folate	-131.2 (-230.1, -32.4)	0.009	-1.0 (-91.3, 89.4)	0.983	-48.4 (-114.0, 17.1)	0.148
Low B ₁₂ ² /Suboptimal Folate ³	157.9 (-83.8, 399.7)	0.200	11.9 (-285.7, 309.6)	0.937	203.9 (-48.8, 456.7)	0.114
Low B ₁₂ ² /Elevated Folate ⁴	70.2 (-288.4, 428.8)	0.701	-382.5 (-658.2, -106.7)	0.007	50.9 (-73.7, 175.4)	0.423

Data presented as mean telomere length (95% CI), significance set at 0.05

¹ Adjusted for age, sex, race, BMI[†], C-reactive protein level[†], homocysteine level[†], educational status, smoking status, alcohol intake

² Low vitamin B₁₂ status defined as serum B₁₂ <148 pmol/L and/or MMA ≥210 nmol/L

³ Suboptimal folate defined as serum folate ≤13.4 nmol/L

⁴ Elevated folate status defined as serum folate ≥45.3 nmol/L

[†] Log-transformed data

Table 2.1:

Characteristics of eligible participants from the National Health and Nutrition Examination Survey (1999-2002) by sex and iron status (N=5,908).

	Men (N = 2933)				Women (N = 2975)			
Continuous Data	Total Men Mean (SE)	Suboptimal Iron Status	Sufficient Iron Status	Elevated Iron Status	Total Women Mean (SE)	Suboptimal Iron Status	Sufficient Iron Status	Elevated Iron Status
Age (years)	50.0 (0.3)	63.3 (1.7)*	49.5 (0.4)	49.3 (0.6)**	50.4 (0.3)	46.3 (0.8)*	50.3 (0.4)	56.2 (0.9)**
Body Mass Index (BMI) (kg/m ²)	27.8 (0.1)	29.2 (0.5)	27.6 (0.1)*	28.1 (0.2)	28.7 (0.1)	30.0 (0.3)	28.4 (0.1)*	28.0 (0.3)
Serum Iron (µg/dL)	95.3 (0.7)	53.9 (3.2)*	89.9 (0.8)**	112.5 (1.1)	81.3 (0.7)	47.6 (0.4)*	84.4 (0.7)**	108.8 (1.6)
Serum Ferritin (µg/L)	158.2 (1.7)	81.6 (7.0)*	111.4 (1.7)**	268.2 (2.5)***	77.0 (1.7)	37.6 (2.6)*	66.0 (1.2)**	187.0 (2.9)***
Transferrin Saturation Percentage (TS %)	27.4 (0.2)	13.7 (1.0)*	25.7 (0.2)**	33.0 (0.3)***	22.3 (0.2)	11.9 (0.4)*	23.2 (0.2)**	31.4 (0.4)***
Erythrocyte Protoporphyrin (EPP) (ug/dL)	47.4 (0.7)	87.1 (1.7)	45.9 (0.4)*	45.0 (0.6)**	61.9 (0.7)	106.0 (2.0)	53.1 (1.0)*	50.9 (2.3)**
Hemoglobin (hb) (g/dL)	15.2 (0.02)	13.6 (0.1)*	15.2 (0.0)**	15.4 (0.0)***	13.5 (0.02)	12.3 (0.0)*	13.7 (0.0)**	13.8 (0.0)***
C-reactive protein (CRP) (mg/L)	3.6 (0.1)	7.7 (0.6)*	3.9 (0.2)**	2.3 (0.2)***	5.1 (0.1)	6.3 (0.4)*	5.1 (0.2)**	3.4 (0.4)***
MCV (fL)	90.5 (0.1)	85.2 (0.4)*	90.5 (0.1)	91.2 (0.2)	89.6 (0.1)	84.7 (0.2)*	90.5 (0.1)	91.3 (0.3)
Hb A1c %	5.57 (0.0)	5.53 (0.1)	5.54 (0.0)	5.64 (0.0)	5.54 (0.0)	5.62 (0.0)	5.51 (0.0)	5.64 (0.0)
Categorical Data	Total Men N (%)	Suboptimal Iron Status	Sufficient Iron Status	Elevated Iron Status	Total Women N (%)	Suboptimal Iron Status	Sufficient Iron Status	Elevated Iron Status
Race								
Non-Hispanic White	1564 (53.3)	67 (4.3)	1022 (65.4)	475 (30.4)	1530 (51.4)	205 (13.4)	1123 (73.4)	202 (13.2)
Non-Hispanic Black	486 (16.6)	24 (4.9)	307 (63.2)	155 (31.9)	511 (17.2)	122 (23.9)	306 (60.0)	83 (16.2)
Mexican/Hispanic	813 (27.7)	25 (3.1)	545 (67.0)	243 (29.9)	848 (28.5)	178 (21.0)	576 (67.9)	94 (11.1)
Multi-Racial	70 (2.4)	1 (1.4)	48 (68.6)	21 (30.0)	86 (2.9)	11 (12.8)	58 (67.4)	17 (19.8)
Education								
Less than 11 th Grade	974 (33.2)	43 (4.4)	614 (63.0)	317 (32.6)	930 (31.3)	197 (21.2)	599 (64.4)	134 (14.4)
High School Graduate	670 (22.8)	23 (3.4)	432 (64.5)	215 (32.1)	727 (24.4)	109 (15.0)	507 (69.7)	111 (15.3)
Some College	666 (22.7)	27 (4.1)	429 (64.4)	210 (31.5)	806 (27.1)	149 (18.5)	566 (70.2)	91 (11.3)
Beyond College	623 (21.2)	24 (3.9)	447 (71.8)	152 (24.4)	512 (17.2)	61 (11.9)	391 (76.4)	60 (11.7)
Income								
Less than \$19K/yr	559 (21.2)	26 (4.7)	373 (66.7)	160 (28.6)	655 (24.9)	131 (20.0)	445 (67.9)	79 (12.1)
\$20K - \$54K/yr	1365 (51.7)	46 (3.4)	874 (64.0)	445 (32.6)	1311 (49.8)	223 (17.0)	905 (69.0)	183 (14.0)
Greater than \$55K/yr	719 (27.2)	30 (4.2)	486 (67.6)	203 (28.2)	666 (25.3)	99 (14.9)	483 (72.5)	84 (12.6)
Smoking Status								
Never Smoker	1283 (43.7)	46 (3.6)	848 (66.1)	389 (30.3)	1862 (62.6)	362 (19.4)	1259 (67.6)	241 (12.9)
Former Smoker	1018 (34.7)	57 (5.6)	659 (64.7)	302 (29.7)	617 (20.7)	89 (14.4)	441 (71.5)	87 (14.1)
<10 cigs/d	145 (4.9)	7 (4.8)	97 (66.9)	41 (28.3)	123 (4.1)	17 (13.8)	87 (70.7)	19 (15.5)
10 – 19 cigs/d	175 (6.0)	1 (0.6)	122 (69.7)	52 (29.7)	159 (5.3)	25 (15.7)	117 (73.6)	17 (10.7)

>=20 cigs/d	312 (10.6)	6 (1.9)	196 (62.8)	110 (35.3)	214 (7.2)	23 (10.8)	159 (74.3)	32 (15.0)
Alcohol Intake								
Never	230 (7.8)	14 (6.1)	157 (68.3)	59 (25.6)	668 (22.5)	132 (19.8)	445 (66.6)	91 (13.6)
Former Moderate	365 (12.4)	24 (6.6)	241 (66.0)	100 (27.4)	501 (16.8)	98 (19.6)	335 (66.9)	68 (13.6)
Former Heavy	210 (7.2)	12 (5.7)	141 (67.1)	57 (27.1)	51 (1.7)	12 (23.5)	34 (66.7)	5 (9.8)
Current Moderate	1173 (40.0)	44 (3.8)	780 (66.5)	349 (29.8)	1348 (45.3)	212 (15.7)	962 (71.4)	174 (12.9)
Current Heavy	955 (32.6)	23 (2.4)	603 (63.1)	329 (34.4)	407 (13.7)	62 (15.2)	287 (70.5)	58 (14.3)
Blood Glucose Status								
Normal	2192 (74.7)	68 (3.1)	1479 (67.5)	645 (29.4)	2253 (75.7)	375 (16.6)	1604 (71.2)	274 (12.2)
Borderline	408 (13.9)	30 (7.4)	260 (63.7)	118 (28.9)	409 (13.8)	72 (17.6)	273 (66.8)	64 (15.7)
Diabetes	333 (11.4)	19 (5.7)	183 (55.0)	131 (39.3)	313 (10.5)	69 (22.0)	186 (59.4)	58 (18.5)

Continuous data presented as unadjusted mean (\pm SD) by sex and iron status

Two sample T-test (*, **, ***) denotes statistically significant differences between men and women by iron status)

Significance set at 0.05

Categorical data presented as N (%) by sex and iron status

Suboptimal iron status defined as ≥ 2 of the following: ferritin level $< 15 \mu\text{g/L}$, EPP $> 80 \mu\text{g/dL}$, TS% < 20 , hb < 12 for women and < 13 for men.

Elevated iron status defined as the following: TS% ≥ 50 or ferritin level $\geq 150 \mu\text{g/L}$ for women and $\geq 200 \mu\text{g/L}$ for men with CRP $< 10 \text{ mg/L}$ or serum iron $\geq 155 \mu\text{g/dL}$ for women and $\geq 160 \mu\text{g/dL}$ for men.

Table 2.2:

Age-adjusted mean telomere length by sex, ethnicity/race and iron status in eligible participants from the National Health and Nutrition Examination Survey (1999-2001).

	Telomere Length by Iron Status in Men (N = 2,933)			
	Total Men N (%)	Suboptimal Iron Status	Sufficient Iron Status	Elevated Iron Status
Race				
Non-Hispanic White	5694.3 (5666.6, 5722.1)	5662.9 (5524.5, 5801.3)	5705.9 (5671.4, 5740.5)	5673.8 (5622.6, 5724.9)
Non-Hispanic Black	5855.5 (5800.8, 5910.3)	6161.2 (5892.9, 6429.4)	5866.3 (5795.8, 5936.7)	5789.9 (5684.6, 5889.3)
Mexican/Hispanic	5658.1 (5621.1, 5695.0)	5750.1 (5523.9, 5976.2)	5661.8 (5615.8, 5707.9)	5640.2 (5569.5, 5710.9)
Multi-Racial	5725.2 (5579.4, 5871.0)	5917.2 (4382.3, 7452.1)	5744.0 (5538.0, 5950.0)	5672.8 (5315.0, 6030.7)

	Telomere Length by Iron Status in Women (N = 2,975)			
	Total Women N (%)	Suboptimal Iron Status	Sufficient Iron Status	Elevated Iron Status
Race				
Non-Hispanic White	5734.5 (5705.6, 5763.3)	5701.1 (5620.6, 5781.5)	5751.4 (5718.1, 5785.7)	5671.6 (5590.5, 5752.7)
Non-Hispanic Black	5943.5 (5888.9, 5998.2)	5973.0 (5854.2, 6091.8)	5918.7 (5846.1, 5991.3)	5991.7 (5844.8, 6138.6)
Mexican/Hispanic	5728.5 (5694.7, 5762.2)	5736.4 (5659.1, 5813.7)	5741.6 (5700.1, 5783.2)	5632.7 (5526.2, 5739.3)
Multi-Racial	5796.7 (5675.5, 5617.9)	5776.5 (5238.0, 6315.0)	5804.0 (5604.4, 6003.5)	5785.1 (5357.6, 6212.7)

Data presented as age-adjusted mean telomere length (95% CI)

Suboptimal iron status defined as ≥ 2 of the following: ferritin level < 15 ng/mL, EPP > 80 ug/dL, TS% < 20 , hb < 12 for women and < 13 for men.

Elevated iron status defined as the following: TS% ≥ 50 or ferritin level ≥ 150 ng/mL for women and ≥ 200 ng/mL for men with CRP < 10 mg/L or serum iron ≥ 155 ug/dL for women and ≥ 160 ug/dL for men.

[†] Log-transformed data

Table 2.3: Multiple linear regression of iron status and telomere length in participants from the National Health and Nutrition Examination

	Model 1 ¹			Model 2 ²			Model 3 ³		
	β , (95%CI) ⁴	p-value	R-sq	β , (95%CI) ⁴	p-value	R-sq	β , (95%CI) ⁴	p-value	R-sq
Iron Status									
Sufficient	-Ref-	---	0.171	-Ref-	---	0.185	-Ref-	--	0.193
Suboptimal	15.7 (-31.9, 63.2)	0.518		-5.9 (-54.0, 42.3)	0.812		2.5 (-45.7, 50.6)	0.787	
Elevated	-48.8 (-84.4, -13.2)	0.007		-41.1 (-77.0, -5.2)	0.025		-36.7 (-72.6, -0.8)	0.045	

¹ Adjusted for age

² Adjusted for age, gender and race/ethnicity

³ Adjusted for age, gender, race/ethnicity, BMI[†], education status, smoking status, and alcohol intake status

⁴ Represents the change in mean telomere length per year

Suboptimal iron status defined as ≥ 2 of the following: ferritin level < 15 ng/mL, EPP > 80 ug/dL, TS% < 20 , hb < 12 for women and < 13 for men.

Elevated iron status defined as the following: TS% ≥ 50 or ferritin level ≥ 150 ng/mL for women and ≥ 200 ng/mL for men with CRP < 10 mg/L or serum iron ≥ 155 ug/dL for women and ≥ 160 ug/dL for men.

[†] Log-transformed data

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