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## The Masking Effect: A comparison of pre and post folic acid fortification periods for vitamin B-12 deficiency without macrocytosis in the United States

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This thesis, *The Masking Effect: A comparison of pre and post folic acid fortification periods for vitamin B-12 deficiency without macrocytosis in the United States*, by Benjamin D. Steele was prepared under the direction of the Master's Thesis Advisory Committee. It is accepted by the Committee members in partial fulfillment of the requirements for the degree Master of Science in the Byrdine F. Lewis School of Nursing and Health Professions, Georgia State University. The Master's Thesis Advisory Committee, as representatives of the faculty, certify that this thesis has met all standards of excellence and scholarship as determined by the faculty.

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## ABSTRACT

**Background:** There has been a concern regarding the masking of vitamin B-12 deficiency in the post-fortification period (after January 1, 1998).

**Objective:** The objective of this study was to investigate the potential masking of vitamin B-12 deficiency by comparing the proportion of individuals with low serum B-12 without macrocytosis between pre- and post-fortification periods using data from the National Health and Nutrition Examination Survey (NHANES).

**Methods:** The study included 7242 individuals from NHANES III (pre-fortification group) and combined NHANES 1999-2000, 2001-2002, 2003-2004, 2005-2006 (post-fortification group). Vitamin B-12 deficiency and macrocytosis were defined as having  $<148$  pmol/L of serum vitamin B-12 and mean corpuscular volume (MCV) of  $>98$  fL, respectively. A multivariate logistic regression was performed to estimate the likelihood of being low serum B-12 without macrocytosis in the post-fortification period in relation to the pre-fortification period.

**Results:** Between pre- and post-fortification periods, there was no significant difference in the proportion of individuals with low serum vitamin B-12 without macrocytosis. However, odds of having low serum vitamin B-12 without macrocytosis in the post fortification era increased in men (OR=2.65, 1.24-5.65), non-Hispanic blacks (OR=3.12, 1.04-9.35), Non-smokers (OR=4.63, 1.90-11.27), and

those aged 55 and older (OR=2.183, 1.01-4.74) compared their respective counterparts in the pre-fortification period.

**Conclusions:** No significant difference in the proportion of individuals with vitamin B-12 deficiency without macrocytosis was seen between the pre and post-fortification periods suggesting no masking of vitamin B-12 deficiency. In the post-fortification period, serum folate was found to be a predictor of the masking effect. The impact of increased folic acid intake in the post-fortification period needs to be evaluated on a periodic basis especially, in non-target population.

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THE MASKING EFFECT: A COMPARISON OF PRE AND POST FOLIC ACID  
FORTIFICATION PERIODS FOR VITAMIN B-12 DEFICIENCY WITHOUT  
MACROCYTOSIS IN THE UNITED STATES

By  
Benjamin Steele

A Thesis

Presented in Partial Fulfillment of Requirements for the Degree of

Master of Science in Health Sciences

Byrdine F. Lewis School of Nursing and Health Professions

Department of Nutrition

Georgia State University

Atlanta, Georgia

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## ABBREVIATIONS

CDC	Center for Disease Control and Prevention
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
FDA	Food and Drug Administration
FLOR1	Folate Binding Protein-1
HS/GED	High School / Graduate Equivalent Degree
IOM	Institute of Medicine
MCV	Mean Corpuscular Volume
MMA	Methylmalonic Acid
MTHFR	Methyl-tetrahydrofolate Reductase
NCHS	National Center for Health Statistics
NHANES	National Health and Nutrition Examination Survey
NTDS	Neural Tube Defects
OR	Odds Ratio
PIR	Poverty Income Ratio
PR	Prevalence Rate
RBC	Red Blood Cell
RNA	Ribonucleic Acid
THF	Tetrahydrofolate
UK	United Kingdom
US	United States
WHO	World Health Organization

## CHAPTER I

### INTRODUCTION

#### Topic and problem

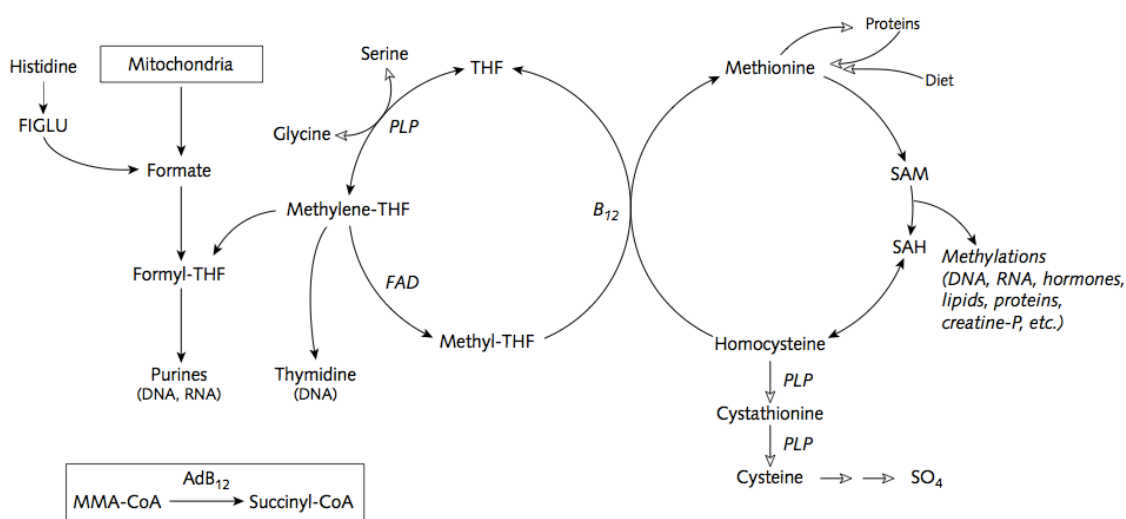
Neural tube defects (NTDs) are linked to folate deficiency in the first trimester of pregnancy, and include occur during the closing of the neural tube of the developing embryo. The cell membranes of the neural crest and neuroepithelial cells of the embryo have high expressions of folate-binding protein 1 (Folr1).<sup>1</sup> This indicates high affinity and demand for folate within these cells. Without adequate folate, these cells do not invaginate and fuse properly.<sup>2</sup> Depending on the specific location of the failed closure, different NTDs occur. Spina bifida is the improper closing of the tail end of the neural tube and results in developmental problems of the spinal column, typically with the spinal cord left unprotected and exposed. Anencephaly occurs when the head of the neural tube fails to close, resulting in improper brain and cranial development. Most babies born with anencephaly do not survive.<sup>3</sup>

Education programs were designed and implemented to increase folic acid consumption among reproductive age females in the United States, however there was little impact on reducing the number of NTD.<sup>3</sup> As a result, and after careful consideration, the Food and Drug Administration (FDA) mandated fortification of processed grains products in the United States with folic acid effective January 1<sup>st</sup> 1998. Other countries, including Chile, Canada, and Australia quickly followed suit. Ultimately these programs are credited with a decrease in the prevalence of NTD by as much as 46%

worldwide.<sup>1</sup> In the United States, the rate of NTD decreased by 19% after fortification. Spina bifida rates in the United States decreased from 26.2 to 20.2 per 100,000 live births, and anencephaly decreased from 11.6 to 10.3 per 100,000 live births.<sup>4</sup> Despite the positive benefits of the fortification programs, the Institute of Medicine identified a potential risk of deleterious neurological effects may increase when a deficiency in vitamin B-12 is masked by increased folic acid consumption.<sup>5</sup> Folic acid and Vitamin B-12 are linked at one conversion reaction within the folate-cycle. At this junction vitamin B-12, also known as cobalamin, acts as a methyl-group transfer molecule and is critical in creating the necessary substrates for methylation reactions. Methylation is an important biochemical process that creates substrates necessary for DNA synthesis, myelination of neurons, regulation of gene expression, and protein synthesis.<sup>6</sup> In this complex, multi-step process, vitamin B-12 and folic acid play a pivotal role in creating the methyl-donor substrate methylene tetrahydrofolate (methylene THF). In its role as a coenzyme with methionine synthase, vitamin B-12 accepts a methyl group from 5-methyl-tetrahydrofolate, which returns to tetrahydrofolate (THF). This process converts cobalamin to methyl-cobalamin, which in turn, donates its methyl-group to homocysteine to form methionine, thus regenerating cobalamin to continue the folate cycle. With vitamin B-12 deficiency, methyl-THF is not reconverted back to THF, causing a breakdown in the cycle, and reduction of the generation of methylene-THF, necessary for DNA synthesis.

Additionally, cobalamin functions as a coenzyme in the conversion of methylmalonyl-CoA to succinyl-CoA, which can enter the Krebs' Cycle to produce energy. During B-12 deficiency levels methylmalonyl-CoA increase, as it is unable to be

converted to succinyl-CoA. Methylmalonyl-CoA spontaneously converts to methylmalonic acid, a functional measure of vitamin B-12 status. The aforementioned cycles do not function properly without adequate folate or vitamin B-12, diminishing the ability to replicate DNA efficiently. This becomes evident with macrocytic anemia, as red blood cells do not have enough genetic material to divide properly, and therefore appear enlarged. Data suggest that an overabundance of folate, in the form of folic acid from fortified foods, may result in a bypass of multi-step, B-12 dependent folate cycle in favor of converting the reduced folic acid from our diet into the necessary 5,10-methyl-tetrahydrofolate, thus preventing the onset of macrocytic anemia.<sup>7,8</sup>



**Figure 1 Folate, B-12 cycle<sup>1</sup>**

<sup>1</sup> Folate, vitamin B-12 and methyl-group transfer cycle. AdB<sub>12</sub>, adenosyl cobalamin; FAD, flavin adenine dinucleotide; FIGLU, formiminoglutamic acid; MMA, methylmalonic acid; PLP, pyridoxal phosphate; SAH, s-adenosyl homocysteine; SAM, s-adenosyl methionine; THF, tetrahydrofolate. Adapted from Selhub et al (2008).

Although successful in reducing NTD, higher consumption of folic acid due to fortification may result in delayed diagnosis of B-12 deficiency as indicated by randomized control studies in animals, and human case and cohort studies.<sup>9,7</sup> Numerous



case studies have shown an increase of neurological complications due to undiagnosed B-12 deficiency.<sup>5</sup> Therefore, increased folic acid consumption may amplify the risk of developing potentially irreversible neuropathology as a result of undiagnosed vitamin B-12 deficiency.<sup>5-7</sup>

### Significance

Previous studies have shown a relationship between elevated serum folate and the prevalence of B-12 deficiency.<sup>7,10-12</sup> However, the literature is mixed as to the masking effect of folic acid. Clarke et al (2008) examined vitamin B-12 status in the United Kingdom, where folic acid fortification is voluntary, not mandatory; the sample population were elderly, and although this population is at higher risk for vitamin B-12 deficiency, it is not representative of the general population.<sup>13</sup> Mills et al (2011) found no evidence suggesting vitamin B-12 masking has occurred in Trinity College students in Dublin, Ireland.<sup>14</sup> Additionally, a baseline investigation of serum folate in Australia found that Aboriginals tend to be folate deficient, yet are B-12 replete, in large part due to minimal consumption of baked flour goods.<sup>15</sup>

Both Vitamin B-12 deficiency and folic acid deficiency are directly linked to the onset of macrocytosis. As folic acid consumption increases through fortification of cereals and processed grain products, the folate-trap that occurs during vitamin B-12 deficiency is no longer relevant. The excess folic acid from fortification is converted to THF and enters the folate cycle to generate the raw materials for DNA synthesis, thus preventing the onset of macrocytosis. Without this clinical manifestation, B-12 deficiency can go undiagnosed until neurological damage occurs. Thus, it seems that folic acid fortification may play a role in masking the clinical diagnosis of vitamin B-12 deficiency.

This may be especially true in those already at risk for vitamin B-12 deficiency, including the elderly, vegetarians, vegans, and pregnant women.<sup>5,16</sup>

### Objectives

A comparison of pre- and post-fortification prevalence of hypovitamin-B-12 without macrocytosis has never performed using population-wide data. Therefore the objective of this study was to investigate if the proportion of those with vitamin B-12 deficiency, presenting without macrocytosis, has significantly increased in the post folic acid fortification period (after January 1, 1998) compared to the pre-fortification period using data from the National Health and Nutrition Examination Survey (NHANES), a nationally representative sample survey.

## CHAPTER II

### REVIEW OF LITERATURE

The reduction of NTDs was the impetus behind folic acid fortification programs across the world. These programs have been heralded as public health success stories due to the significant reduction of neural tube defects. However, the positive impact of these programs has since been met with controversy: unintended consequences, including the masking of vitamin B-12 deficiency.

#### Impact of folic acid fortification on the prevalence of NTDs

Honein et al (2001) performed one of the first post-fortification period analyses on the impact of the folic acid fortification program with the prevalence of neural tube defects in the United States. The study included birth certificate reporting of spina bifida or anencephaly from 40 states from January 1990 through December 1999. A pre-fortification population from 1990-1996 was used as a baseline measure of the prevalence of NTD. However, a more specific time-dependent group from five quarters immediately before and five quarters immediately following fortification were used for direct comparisons. Prevalence of spina bifida reported from October 1995 through December 1996, representative of the pre-fortification period, was 26.2 per 100,000 live births. During the post fortification period observed, the prevalence of spina bifida decreased to 20.2 per 100,000 live births, a reduction of 23% (PR, 0.77; 95% CI: 0.70, 0.84). Prevalence for anencephaly was 11.6 per 100,000 live births in the pre-fortification

period and dropped to 10.3 per 100,000 live births, representing an 11% decline (PR, 0.89; 95% CI: 0.78, 1.01). Total NTDs saw a 19% reduction (PR, 0.81; 95% CI: 0.75, 0.87) in prevalence in the post-fortification period observed in this study. A relatively consistent, long-term downward trend in the prevalence of anencephaly was seen prior to the mandatory fortification in 1998, however, the authors cautioned about a direct attribution of folic acid fortification with the decrease in anencephaly during the post-fortification period. This study only included live births, and it did not account for spontaneous or induced abortions, which might have caused an underreporting of true incidence and prevalence for NTD.

Boulet et al (2008) analyzed data from the National Birth Defects Prevention Network NTD Ascertainment Project from 1999-2004 in order to establish a trend in NTD during the post-fortification period.<sup>1</sup> A total of 3,311 cases of spina bifida and 2,116 cases of anencephaly were reported. A significant decrease of 10% (PR, 0.90; 95% CI, 0.84-0.96) was seen for the combination of spina bifida and anencephaly from 1999-2000 and 2003-2004. However, when taken alone, the decline in spina bifida was found to be insignificant (PR 0.97; 95% CI: 0.89, 1.05), whereas the decline in anencephaly was significant (PR 0.80; 95% CI: 0.72, 0.89). The authors postulated that the prevalence rate of spina bifida might plateau at these lower levels as serum folate levels have improved, approaching the limit of folic-acid-preventable defects.

Interestingly, Mosley et al (2008) were unable to correlate folic acid supplementation or dietary folate intake with NTD after analyzing data from the National Birth Defects Prevention Study from 1998-2003, a case-control study. Cases that reported not using supplements did not differ from non-supplement using controls with

consumption of dietary folate. Additionally, similar results were found for women who used supplements between 3 months prior to pregnancy through the first month of pregnancy compared to those who initiated supplement use after the first month of pregnancy. The authors postulate that the sample population may have had sufficient folate status as to be protective against folate-dependent neural tube defects, and cases could be the result of other factors beyond folate consumption. Although supplementation use and dietary folate did not correlate, significant differences between cases and controls existed for race-ethnicity, education, and income. Cases were more likely to be Hispanic, less likely to report education beyond high school, and less likely to report income at or beyond \$50,000. Anencephaly taken alone was associated with a higher likelihood of reporting smoking or alcohol use during pregnancy when compared to controls. For spina bifida alone, cases were more likely to have a BMI  $\geq 30\text{kg/m}^2$  and less likely to report the pregnancy as intended than controls.

Thompson et al (2009) assessed the literature on NTD in Canada with regards to serum folate and vitamin B-12 status. Folic acid fortification was found to have a marked reduction on the prevalence of NTD in Ontario based on the analysis of 336,963 women between the pre- and post-fortification periods. The prevalence dropped from 113/100,000 pregnancies during the pre-fortification period to 58/100,000 pregnancies post-fortification (PR 0.52; 95% CI, 0.40, 11.0). For vitamin B-12 status in the lowest quartile, as measured by holotranscobalamin, the odds ratio of a NTD was 3.2 (95% CI: 0.94, 11.0) and the population attributable risk was as high as 35%. This indicates that low vitamin B-12 status may play a role in NTD prevalence. Data suggest that as many as 5% of women of childbearing age in Ontario have low vitamin B-12 status. The authors

suggested the development of a randomized controlled trail to investigate the impact of low vitamin B-12 status on the development of NTDs.

In their review, Obican et al (2010) looked at the literature surrounding our understanding of genetic impacts on enzyme function in the folate pathway as well as epidemiologic investigations into the role of folic acid embryonic development. Most notably within the genetic sphere of folate metabolism is the impact folate-binding protein 1 (Folr1) plays on embryonic development. Data suggest that this membrane-bound receptor is expressed more on neural crest and neuroepithelial cells during development, and therefore it plays a significant role in the link between folate and NTD. Knocking out this gene in mice resulted in severe developmental abnormalities, often leading to spontaneous abortion. However, when supplemented with folic acid, spontaneous abortions were reduced, but malformations persisted, including those in the formations of the neural tube, cardiovascular system, and lip and palate structures. Additionally the role of Pcf1/Hcp1, a transporter found in the intestines and involved with both heme and folate absorption, was strongly associated with the development of macrocytic anemia once knocked out in mice. Methyl-tetrahydrofolate reductase polymorphisms were shown to be a genetic risk factor for NTD. As for fortification programs, the review found a worldwide reduction of 46% (95% CI: 0.37, 0.54) in the prevalence of NTD as a result of fortification programs. In the United States, an overall reduction in NTD of 19% has been observed. As a result, these programs have been deemed a public health success, despite potential adverse effects related to vitamin B-12 deficiency masking, and the increase of colorectal cancer incidence by an absolute

incidence of 4-6 cases/100,000 individuals during the immediate post-fortification years.<sup>19</sup>

### Measuring vitamin B-12 and folate status

Lack of consensus in the definition vitamin B-12 deficiency has been the most difficult aspect in the investigation into the potential masking effect posed by increased folic acid consumption as a result of grain fortification. No gold standard yet exists for the measurement and definition of vitamin B-12 deficiency, and various levels for serum B-12 concentrations are used throughout the literature as the cut point to indicate deficiency. The range for serum B-12 concentration cut off points fall as low as 74 pmol/L to as high as 258 pmol/L. Measuring indirect functional indicators versus direct serum concentrations can also be problematic as age, gender, and medical conditions can alter these values and hamper diagnosis of vitamin B-12 deficiency.

In 2008 Selhub et al set out to establish consensus with regards to direct measurement of plasma or red blood cell concentrations of folate and vitamin B-12, versus the measurement of functional indicators such as mean corpuscular volume (MCV), serum methylmalonic acid (MMA), and serum homocysteine. However, the authors noted the difficulty in establishing a standard as both direct concentrations and functional indicator concentrations change with age and gender. Serum folate levels are nearly twice as high for those older than 60 years versus those aged 12-19 years. A similar trend exists for red blood cell folate. The opposite is true for serum vitamin B-12 concentrations: a decrease in concentration is seen with an increase in age. Establishing an optimal plasma folate, serum B-12 or red blood cell folate concentration that is applicable for everyone is almost impossible because of fluctuations that occur across the

age spectrum and differences attributable to gender. The use of these direct measures may not be indicative of a true vitamin deficiency. Functional indicators for folic acid are a combination of homocysteine and methylmalonic acid, while the best functional indicator for vitamin B-12 is MMA. Since cellular homocysteine homeostasis is tightly controlled, excess homocysteine ends up in blood; however, plasma homocysteine increases with age, and is found at higher concentrations in men than women. The researchers found a biphasic relationship between serum vitamin concentrations and their functional indicators, and therefore they constructed two-phase regression models to determine at what plasma concentration the functional indicators achieve a minimal optimal level. Serum folate concentrations around 10 nmol/L achieved optimal homocysteine concentrations as indicated by the flattening of the biphasic curve. Red blood cell folate concentration of 340 nmol/L produced the optimal level of homocysteine. For serum vitamin B-12, a concentration of 150 pmol/L established the minimal optimal level of MMA, however a concentration of 300 pmol/L was found for homocysteine. Based on data from the regression analysis, a cut point of serum vitamin B-12 concentration of 150 pmol/L is in accordance with other clinical findings; however the authors suggest the use of 300 pmol/L for portions of the population, like the elderly, where vitamin B-12 deficiency occurs at a higher rate.

Ray et al (2000) performed analysis on data obtained from MDS Laboratories of Ontario on individuals who had serum homocysteine, RBC folate, and serum vitamin B-12 measures taken over an nine month period of time in 1999. The data from a total of 692 individuals met the criteria for inclusion for analysis. A concentration of <120 pmol/L was set as low vitamin B-12 status, and between 120-150 pmol/L as



indeterminate vitamin B-12 status. The geometric mean levels for serum and RBC folate, as well as serum vitamin B-12, were higher for females compared to males in this population. A geometric mean of serum homocysteine was significantly higher in males (9.3  $\mu\text{mol/L}$ ) than in women (8.3 $\mu\text{mol/L}$ ). Elevated homocysteine ( $>15 \mu\text{mol/L}$ ) was found to be a poor indicator of vitamin B-12 deficiency as the probability of having serum vitamin B-12 concentration  $<120 \text{ pmol/L}$  in the presence of elevated homocysteine levels was 7.4%. Thus the authors advise the use of caution when using homocysteine levels as a sole indicator of vitamin B-12 deficiency.

#### Folate and B-12 status in countries with mandatory fortification

Ray et al (2008) performed a retrospective cross-sectional study to determine prevalence of vitamin B-12 deficiency in Canadian women of reproductive age. This study took place nearly a decade after Canada mandated folic acid fortification. Serum vitamin B-12 concentrations below 125 pmol/L were set as indicating vitamin B-12 deficiency. A significant difference in mean serum vitamin B-12 concentration was associated with stage of pregnancy ( $\leq 28$  days, indicating neural tube closure had yet to occur; and  $>28$  days, suggestive of neural tube closure). Vitamin B-12 deficiency occurred in 6.9% of non-pregnant women, 5.2% of women pregnant  $\leq 28$  days, and 10.1% of women pregnant  $>28$  days. This last group of women was found to be 40% (95% CI: 10-80) more likely to be deficient in vitamin B-12 than non-pregnant women.

Ganji and Kafai (2012) conducted an epidemiological investigation into the prevalence and attributable risk of elevated serum MMA, a sensitive functional measure of vitamin B-12 status, in the post-fortification period. Using data from three cycles of

NHANES (1999-2004), a final sample size of 18,238 was obtained. Elevated MMA was defined as  $>350$  nmol/L. Serum creatinine was used as a determinant for kidney function and set at  $\geq 130$   $\mu$ mol/L to indicate kidney dysfunction. Serum vitamin B-12 concentrations  $<148$  pmol/L was used to establish vitamin B-12 deficiency. For this sample population, age, race-ethnicity, supplement use, and serum vitamin B-12 concentration were linked to elevated MMA. The likelihood of having elevated MMA was found to be significantly higher in those with serum vitamin B-12 concentrations  $<148$  pmol/L (PR, 27.6%; OR, 13.5;  $P < 0.0001$ ), as well as in those who were  $\geq 60$  years of age (PR, 8.3%; OR, 4.0;  $P < 0.0001$ ), and in non-supplement users (PR, 3.1%; OR, 1.93;  $P < 0.0001$ ). The population attributable risk percentage for elevated serum MMA was strongly associated with age at nearly 41% for those aged 60 and older versus those aged less than 60 years; with non-Hispanic white race-ethnicity at nearly 25% compared to all other ethnicities combined; and with those who had serum vitamin B-12  $<148$  pmol/L at roughly 16%. Factors that increase the likelihood of having elevated serum MMA include being non-Hispanic white, being older than 60, having low vitamin B-12 status, and not using supplements. The prevalence of low vitamin B-12 status was 2.0%. Overall, an estimated reduction of about 5 cases of elevated serum MMA per 1000 people would be eliminated if vitamin B-12 status increased to  $\geq 148$  pmol/L.

Australia introduced mandatory folic acid fortification for wheat flour in 2009, but the country has had a voluntary fortification policy established since 1995. Maxwell et al (2012) set out to establish the baseline of folate status in aboriginal and non-aboriginal Western Australians immediately prior to mandatory fortification. The participants included 191 Aboriginal and 159 non-aboriginal Australians. The cutoff for

folate deficiency was set at <250 ng/mL for the assay used, which equates to 140 ng/mL used by the BioRad assay kit used by NHANES. The cutoff for vitamin B-12 deficiency was set at 200 pg/mL. The majority of participants reported not using vitamin supplements. All participants were found to be vitamin B-12 replete. Furthermore, 10% of aboriginal women (95% CI; 5, 19), and 26% of aboriginal men (95% CI; 2, 12) had RBC folate levels below 250 ng/mL, while no non-aboriginal women (95% CI; 0, 4) and only 4% of non-aboriginal men (95% CI; 2, 12) fell below 250 ng/mL for RBC folate. The higher rate of suboptimal folate status in aboriginal women is in line with other studies that found increased prevalence of NTDs in the aboriginal population compared to the non-aboriginal population.

#### Post-fortification era evidence for vitamin B-12 masking

Mills et al (2003) analyzed data from 1,573 patients at the Veteran's Affairs Medical Center in Washington, DC between 1992 and 2000 to determine whether folic acid fortification increased the proportion of those with low vitamin B-12 status (defined as <258 pmol/L) without macrocytic anemia. There were 1573 subjects who had both serum B-12 and MCV measured: 96.1% were male, 69% were African American, and the median age was 67 years. The proportion of those with low vitamin B-12 status without megaloblastic anemia (defined as MCV >96.7 fL) for the pre fortification period was 39.2%. For the peri-fortification and post-fortification periods the proportions were 45.5% and 37.6%, respectively. There was no statistically significant change in proportions of low serum vitamin B-12 without anemia from the pre- to the post-fortification period (OR 0.95; 95% CI; 0.74, 1.22; P = 0.67). When the limit for low vitamin B-12 was adjusted downward to <150 pmol/L, no significant difference between

pre and post-fortification periods was found for those with low vitamin B-12 status without megaloblastic anemia. However, nearly 60% of cases with initial low vitamin B-12 status presented with macrocytosis. Interestingly, the data suggest the likelihood of low vitamin B-12 status is greater in those <60 years of age than for those greater than 60 years ( $P < 0.0001$ , logistical regression).

Pfeiffer et al (2005) reviewed NHANES III and NHANES 1999-2000 data to establish the effect of folic acid fortification on biochemical indicators of B-12 status including serum B-12, MMA, serum homocysteine, serum folate, and red blood cell folate. They note that low serum folate in conjunction with low vitamin B-12 status and an elevated homocysteine level is associated with the development and progression of dementia and Alzheimer's disease, as well as other conditions of cognitive dysfunction: declined psychical functioning; psychiatric disorders; and increased risk of carotid artery stenosis. In their analysis they found a significant upward shift in serum folate across the population, a moderate increase in RBC folate, and a slight increase in serum vitamin B-12. Prevalence of low serum folate (set at  $<6.8$  nmol/L) decreased by 16%; low RBC folate ( $<317$  nmol/mL) decreased by 31%; and vitamin B-12 deficiency (set at  $<74$  pmol/L) "moderately low serum B-12" ( $<185$  pmol/L) remained unchanged at 1% and 5% respectively, Since initiation of fortification in the United States, a shift to higher serum folate concentrations has occurred.. This trend is higher than the expected range of 70-140  $\mu\text{g}/\text{d}$  of folate as anticipated by the US fortification program. Based on the cutoff values established in this study,  $<1\%$  had serum vitamin B-12 concentrations met the definition of deficiency, whereas 5% of the sample had vitamin B-12 levels below 148 pmol/L, indicating moderately low vitamin B-12 status. Additionally, the prevalence of

moderately low serum vitamin B-12 concentration decreased from the pre-fortification period to the post-fortification period in the elderly population. The authors noted the lack of evidence, suggesting an increased risk of B-12 masking. However, due to the limited passage of time between pre-fortification and post-fortification measurements presented in this study, they suggested inclusion of additional NHANES survey periods in the analysis to establish if the trend of vitamin B-12 masking exists.

Clarke et al (2008) investigated the relationship of high serum folate and low B-12 status with cognitive impairment and anemia in 2,476 elderly persons in the United Kingdom (UK). Subjects included two cohorts: one from before and one from after voluntary folic acid fortification. Low vitamin B-12 status was established for this study at concentrations of holotranscobalamin (holoTC)  $<45$  pmol/L. Serum folate  $>30$  nmol/L was considered a high level of concentration. Anemia was defined as hemoglobin  $<12$  g/dL in men, and  $<11$  g/dL in women. Total serum folate levels increased between the two groups measurements; however, B-12 levels remained fairly constant. An increased risk of anemia was observed amongst those in the lowest tertile for both serum folate (OR 2.02; 95% CI; 1.46, 2.80) and holoTC concentrations (OR 1.87; 95% CI; 1.44, 2.43), respectively, as compared to those in the upper tertile of each biochemical marker. Additionally, increased risk of cognitive impairment was seen in the lowest tertiles for folate (OR 1.55; 95% CI; 1.35, 2.12) compared to the upper tertile, and for the lowest tertile of serum vitamin B-12 (OR 1.87; 95% CI; 1.44, 2.43) compared to the upper tertile for vitamin B-12. There was no significant difference in mean homocysteine or MMA concentrations in those with low vitamin B-12 status when stratified by normal and high serum folate levels. Additionally, no significant difference in the risk of cognitive

impairment was found in those with low serum vitamin B-12 and normal serum folate (OR 1.5; 95% CI; 0.91, 2.56) versus elevated serum folate (OR 1.45; 95% CI 1.19, 1.76). No difference in risk of anemia was found between those with low vitamin B-12 status and normal serum folate versus high serum folate concentrations. These analyses indicate that an increased level of serum folate in conjunction with low vitamin B-12 status does not increase the risk of cognitive impairment in elderly UK citizens. The authors speculate that the increase in serum folate in the UK has occurred more recently compared to the United States; therefore additional time may need to elapse to show the true relationship between elevated serum folate and low vitamin B-12 status, and cognitive impairment.

Mills et al (2011) investigated the effects of high folate concentrations on vitamin B-12 status of 2,507 students attending Trinity College in Dublin, Ireland. Non-fasting blood samples were obtained to analyze serum folate, RBC folate, serum vitamin B-12, MMA, and plasma homocysteine levels. Methyl-tetrahydrofolate reductase (MTHFR) genotyping was also performed. Anemia was defined  $<13.0$  g/dL in men and  $<12.0$  g/dL in women. Macrocytosis was defined as an MCV  $\geq 99$  fL. Serum holoTC were deemed low if  $<20$  pmol/L. Elevated homocysteine was set at  $>15$   $\mu\text{mol/L}$ ; elevated serum MMA concentration was defined as  $>0.26$   $\mu\text{mol/L}$ ; elevated serum folate was  $>30$  nmol/L; and low serum vitamin B-12 status was set at a cut off of 148 pmol/L. Participants were grouped according to serum vitamin B-12 concentration (above or below 148 pmol/L) and serum folate status (above or below 30 nmol/L). Group 1 (n=43) consisted of those with low vitamin B-12 status and elevated serum folate, and Group 2 (n=85) consisted of those with low vitamin B-12 status and normal folate status. As expected those that tested

positive for the MTHFR gene variant had significantly lower serum and RBC folate ( $P < 0.001$ ) and higher concentrations of homocysteine ( $P < 0.001$ ). After adjusting for confounds for age, sex, serum ferritin, and plasma creatinine, MTHFR was not a significant predictor of anemia (OR 0.483; 95% CI; 0.130, 1.946;  $P = 0.3$ ). No subjects in either group displayed macrocytosis. No participants in Group 1 had both elevated homocysteine and MMA levels, whereas nine participants in Group 2 did (10.6%). The results of this study do not support the claim that elevated serum folate interferes with vitamin B-12 metabolism in those who are vitamin B-12 deficient.

Hirsch et al (2002) designed a prospective study to investigate the immediate impact of mandatory folic acid fortification on an elderly population in Chile. Based on the evidence of folic acid's impact of reducing the prevalence of neural tube defects, Chile mandated folic acid fortification starting in 2000. A total of 108 participants agreed to the study. The subjects had fasting blood samples taken in December 1999, before the mandatory fortification, and again in July 2000, after the mandate had taken effect. Both serum folate and serum vitamin B-12 levels were positively skewed at baseline, whereas homocysteine was normally distributed. Serum folate levels increased from the pre- to post-fortification periods from 16.2 nmol/L to 32.7 nmol/L ( $P < 0.001$ ). Serum homocysteine levels were reduced by 11.7% in the post-fortification period from 12.95 nmol/L to 11.43  $\mu$ mol/L ( $P < 0.001$ ). Nearly one-third of participants had "subclinical" vitamin B-12 deficiency (defined as serum vitamin B-12  $<165$  pmol/L) at baseline with no improvement at the second measurement. The authors concluded that with prolonged vitamin B-12 deficiencies and improved folate status a masking effect

could occur in the future. Therefore, the authors stated that fortification of grains with folic acid might not be suitable for an elderly population.

In their critical review Selhub and Paul (2011) indicated that folic acid consumption increased to more than 200  $\mu\text{g}$ , well above the predicted 140  $\mu\text{g}$  increase estimated by the Food and Drug Administration (FDA). Additionally a larger percentage of the population, as determined by analysis done on the Framingham cohort, consumed more than the FDA's Upper Limit recommendation of 1000  $\mu\text{g}$  per day, calculated by the lowest observed adverse effect level multiplied by a factor of five. In NHANES data analysis, those with low vitamin B-12 status and serum folate levels  $>59$  nmol/L were observed to have a nearly two-fold increase in prevalence of anemia and cognitive impairment. Un-metabolized folic acid present in the blood is associated with diminished cognitive test scores in seniors with low serum vitamin B-12 levels. The authors argue that these findings suggest high serum folate levels exacerbate both biochemical and clinical statuses for those with vitamin B-12 deficiency.

Morris et al (2007) analyzed NHANES data from two cycles (1999-2000, 2001-2002) of the survey for the effect of folic acid fortification in relation to anemia, macrocytosis, and cognitive impairment in older Americans. Cut off levels were set for anemia at  $<12$  g/dL for women and  $<13$  g/dL for men and in accordance with the World Health Organization criteria;  $\text{MCV} \geq 99$  fL; low serum vitamin B-12 at 148 pmol/L; elevated serum MMA  $>210$  nmol/L; and high folate status as  $>59$  nmol/L. Excluded were those with elevated serum creatinine levels ( $>131$   $\mu\text{mol/L}$  for men and  $>115$   $\mu\text{mol/L}$  for women) as indicative of renal dysfunction; recent anemia therapy; history of stroke; heavy alcohol use; and kidney, liver, or coronary artery diseases. Seniors with low



vitamin B-12 status in conjunction with elevated serum folate levels represented approximately 4% of the sample of 1,684 eligible participants. Among those with normal vitamin B-12 status, high serum folate concentrations had a protective effect for cognitive impairment. Low vitamin B-12 status regardless of folate concentration was associated with increased prevalence of anemia and cognitive impairment. However, the strongest association for increased cognitive impairment was seen in those classified as low vitamin B-12 status with high serum folate concentration (OR: 2.6; 95% CI: 1.1, 6.1). Anemia was observed nearly a five times more often in those with low vitamin B-12 and high serum folate levels as compared to those in normal vitamin B-12 and folate groups. Elevated homocysteine levels were significantly lower in the group with low vitamin B-12 status and normal folate concentrations versus elevated folate concentrations. This study found that high levels of serum folate provided a protective effect against cognitive impairment for vitamin B-12 replete older individuals, but in individuals with low vitamin B-12 status, elevated serum folate advanced the onset or progression of cognitive impairment in line with the neurological manifestations associated with vitamin B-12 deficiency.

Wyckoff and Ganji (2007) investigated the change in proportion of what? those with low serum vitamin B-12 (defined as  $<258$  pmol/L) without macrocytosis (defined as  $MCV >96.7$  fL). Medical records at Rush University Medical Center from individuals  $\geq 19$  years of age who had serum vitamin B-12 and MCV measured between January 1995 and December 2005 were examined. Since the span of time incorporated the folic acid fortification date in 1998, pre and post-fortification data could be compared. A total of 633 records were identified that met the inclusion criteria, and subjects were stratified

into three groups: pre-fortification, peri-fortification, and post fortification. After analysis, MCV was found to be significantly lower in the post-fortification period compared to the pre-and peri-fortification periods for gender ( $P < 0.001$ ) and race-ethnicity ( $P < 0.001$ ). Of those with low vitamin B-12 concentration, the proportion of those without macrocytosis increased in each stratified group: 70%, 85%, and 87% respectively, and each were significantly different ( $P < 0.001$ ). During the post fortification period, the odds ratio for low vitamin B-12 status without macrocytosis, when adjusted for age, gender, and race, was three-times greater for the post-fortification period, compared those in the pre-fortification period. This suggests that an increase in the proportion of those with low serum B-12 without macrocytosis in the post-fortification era.

## CHAPTER III

### METHODS

#### Data Acquisition

Data from NHANES III, during the pre-fortification period, and three surveys from the post-fortification period, NAHNES 1999-2000, NHANES 2001-2002, and NHANES 2003-2004 and NHANES 2005-2006, were used to determine if the prevalence of B-12 deficiency without hematological manifestations has increased during the post-fortification period. This data is from publically available databases, maintained and released by the National Center of Health Statistics (NCHS), and a detailed description of each survey's methodologies and analytic guidelines were reported elsewhere, but a brief description of each survey follows.<sup>23,24</sup> The NHANES III, a periodic survey conducted between October 1988 and October 1994, and using a complex, multi-staged, stratified, clustered sample, consisted of 36,995 participants. Of those, 33,994 individuals were interviewed in their home. A total of 30,818 participants were examined by trained medical professionals in Mobile Examination Centers (MEC) and an additional 493 had examinations performed in their home. After the NHANES III, data collection switched to annual surveys. This study included four cycles of the continuous annual NHANES series (1999-2000, 2001-2002, 2003-2004, and 2005-2006). The NHANES 1999-2000 was conducted between March 1999 and December 2000, and included 9,965 individuals, all interviewed in-home, and 9,282 examined in MECs; NHANES 2001-2002, conducted between January 2001 and December 2002, was comprised of 11,093 individuals, all

interviews conducted in-home, and 10,477 examined in MECs; NHANES 2003-2004, conducted between January 2003 and December 2004, was comprised of 12,761 individuals, 10,122 interviewed in-home, 9,643 examined in MEC. The NHANES 2005-2006 was conducted between January 2005 and December 2006, and consisted of 12,862 individuals. A total of 10,348 were interviewed in-home, and 9,950 were examined in MECs. Taken together, the continuous NHANES combined sample population was 57,158, and 39,352 medically examined individuals. All blood samples were taken with consent, by venipuncture following standard protocol either during the home examination, or in the MEC. The NHANES 1999-2005 surveys a quantitative, automated hematology analyzer was used to measure MCV (Coulter method), serum B-12 and serum folate were measured by using the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay kit. NHANES III utilized a previous generation kit, the Bio-Rad Laboratories "Quantaphase Folate" radioassay kit.

#### Variable Selection

This study focuses on the masking effect of folic acid fortification on low vitamin B-12 status as evidenced by low serum B-12 and without macrocytic anemia. The serum concentrations of primary concern in this investigation were serum folate, serum vitamin B-12, and MCV. The standard definition of macrocytosis is a MCV >98 fL for both men and women. Although no standard has been established for a specific concentration of B-12 that signifies deficiency, a concentrations <148 pmol/L was considered to be deficient in accord with other studies, and the roundtable for B-12 biomarkers in NHANES.<sup>8,25,26</sup> These data were collected during the medical examination portions of each NHANES data collection period.

Demographics of interest and assessed for significance were race/ethnicity, age, gender, education, and PIR. Additional covariates analyzed were alcohol consumption, smoking status, and serum creatinine. Excessive alcohol intake can result in an increase in MCV through interference with folate metabolism.<sup>27</sup> Elevated serum creatinine is an indicator of kidney dysfunction, which can result in the development of anemia. This occurs due to decreased ability of the kidneys to synthesize erythropoietin, the red blood cell production stimulation hormone.<sup>6</sup> Smoking has been linked to disruptions in the B-12 metabolic pathway, primarily in the form of elevated homocysteine levels.<sup>28</sup> These data were collected during the examination and questionnaire portion of the study, except for creatinine, which was collected during the medical examination. From the analysis of the NHANES data, individuals with missing data for any variable were excluded from analysis.

### Recoding Data

Data from NHANES III and the Continuous NHANES series have different variable names, and different reporting formats. Therefore data had to be cleaned and reformatted in order to be comparable. Demographic data from the continuous NHANES set for race/ethnicity, and education were recoded to reflect the data from NHANES III. The upper cutoff value for reporting age shifted down from NHANES III to the continuous NHANES series, from 90 years to 85 years. This resulted in recoding any age >85 in the NHANES III set to equal 85. Education reporting varied from each era, and was recoded as categorical data representing those who did not graduate high school, those identifying as only a high school graduate and, lastly, those who identified as being a college graduate. PIR was reported differently in both versions of NHANES and

required recoding. The upper cutoff value for PIR in the continuous NHANES was set at five, where NHANES III calculated the precise ratio. Thus, the data from NHANES III were recoded so that any data point for  $PIR > 5$  would be equal to five, in accordance with the reporting system of the continuous NAHNES. Alcohol consumption was tallied from the self-reported average number of alcoholic drinks per day and recoded as an average daily intake of less than or equal to one drink, two to three drinks, and greater than three drinks. Smoking was recoded a dichotomous categorical variable, 'yes' or 'no,' based on current smoking status. Those that reported as "smoke sometimes" in the continuous NHANES series were considered as smokers.

A new variable was created using the data for MCV and serum B-12 concentrations to establish a marker for the masking effect. Those individuals who met the criteria for low serum B-12 ( $< 148$  pmol/L) in addition to normal MCV levels ( $< 98$  fL) were flagged as "masking effect present" for the purpose of this study. All other individuals who did not meet these two criteria were flagged as "no masking effect present." Lastly, a new variable was assigned to each data set establishing from which period the data originated. This "pre/post" variable was created and assigned prior to the merging of NAHNES III and continuous NHANES data sets, where a value of zero represented the pre-fortification data, and a value of one represented the post-fortification data. Once all variables were properly coded, and new variables assigned, the separate data sets were merged into one for final analysis. Additional variables were created from stratifying age into four groups:  $\leq 19$  years, 20-39 years, 40-59 years, and  $> 60$  years old. A new variable for PIR was stratified into categories based on the quartiles for the

continuous variable. The four categories represented quartile ranges for PIR from  $\leq 1.99$ , 1.2-2.37, 2.38-4.21 and  $>4.22$ .

### Statistical Analysis

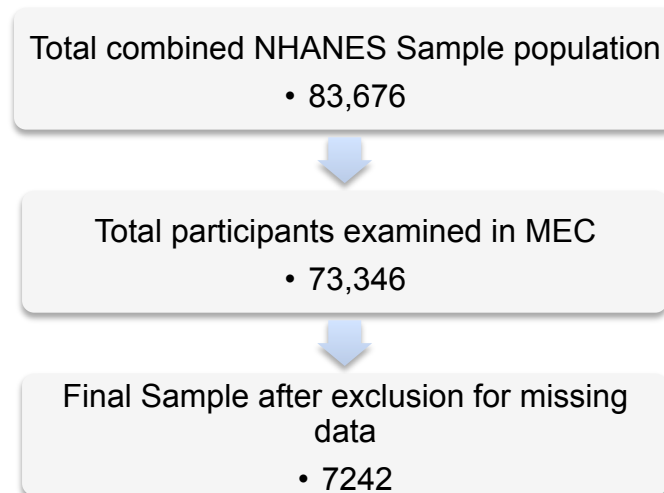
Univariate analysis was conducted for each independent variable in order to determine if any were significant predictors of serum B-12 status, and the distribution of each variable. A non-parametric two-sample test was performed to establish differences between the distributions of each variable for pre and post-fortification groups. The Kolmogorov-Smirnov two-sample test (D) was used to determine differences between the pre- and post-fortification groups for the continuous data. This test was chosen as it is robust to outliers and is more sensitive to the change in shape and distribution than the Mann-Whitney test. Any variables that were significant at  $\alpha=0.05$  were then combined to test a full model of B-12 masking. Finally a logistic regression was performed in order to establish the likelihood of the masking effect occurring in the post-fortification era. These data were analyzed using SAS 9.2 statistical software (SAS Institute Inc, Cary, North Carolina.)

## CHAPTER IV

### RESULTS

After scrubbing for missing data for demographics (age, gender, race/ethnicity, and education), serum concentrations (folate, creatinine, B-12) and additional confounders (PIR, smoking status, and alcohol consumption) over 90% of the individuals from the combined data set (n= 76,840) were removed, leaving data from a total of 7,242 individuals included in this study. The process for selection of the study sample is depicted in **FIGURE 2**. The population was comprised of 61% males (n=4,395) and 39% females (n=2847), who were an average age of  $45 \pm 17.6$  ( $\pm$ SD). The demographic characteristics for the sample population are shown in **TABLE 1**.

**Figure 2 Sample Population Funnel Selection**





From the pre-fortification group, comprised of the NHANES III sample population, data from a total of 1,936 individuals were included in the analysis. The majority of this population (62.5%) were males (n=1,210) and the average age of the participant was 41 years, with a range of 17-85 years old. Two-thirds of the pre-fortification era sample population received a high school education or less. For the PIR, the mean was 2.38, and the median was 2.02. Non-Hispanic whites made up the largest proportion of individuals at 42% (n=815). Only 35 individuals in the pre-fortification period had serum B-12 levels below 148 pmol/L while maintaining normal MCV levels. This represents 1.8% of the sample population. The weighted sample of the masking effect represents over five hundred thousand individuals, and comprises 2.05% of the weighted sample population.

From the combined NHANES 1999-2006 a total of 5,306 individuals had all variable elements required for analysis. This sample was comprised of 60% males (n=3,185) and 40% females (n=2,121). The average age of the interviewee was 45 years old, a range of 20-85 years. The proportion of those who graduated college increased in the post-fortification period compared to the pre-fortification. When PIR was taken as a continuous variable, a significant increase was seen among those in the post-fortification period (Kolmogorov-Smirnov D 3.86,  $P < 0.001$ ). The masking effect was present in 129 individuals in this group, representing 2.43% of the sub-sample population. When the sample weight was taken into account, this sample represents nearly 1.5 million individuals at 2.32% of the weighted sample population of over 63 million.

Table 1 Demographics by fortification period

Table of Demographics by fortification period			
	Fortification Period		
	Pre n = 1,936	Post n = 5,306	Total n = 7,242
<b>Gender, n (%)</b>			
Male	1,210 (62.5%)	3,185 (60.03%)	4,395 (60.69%)
Female	726 (37.5%)	2,121 (39.97%)	2,847 (39.31%)
1-19 yrs	47 (2.43%)	0	47 (0.65%)
20-39 yrs	896 (46.28%)	1,915 (36.09%)	2,811 (38.82%)
40-59 yrs	580 (29.96%)	1,783 (33.6%)	2,363 (32.63%)
60+ yrs	413 (21.33%)	1,608 (30.31%)	2,021 (27.91%)
55+ yrs	1,423 (73.50%)	3,395 (63.98%)	4,818 (66.53%)
≤55 yrs	513 (26.50%)	1,911 (36.02%)	2,424 (33.47%)
<b>Race/Ethnicity, n (%)</b>			
NH white	815 (42.10%)	3,111 (58.63%)	3,926 (54.21%)
NH black	553 (28.56%)	829 (15.62%)	1,382 (19.08%)
Hispanic	509 (26.29%)	1,200 (22.62%)	1,709 (23.60%)
Other	59 (3.05%)	166 (3.13%)	225 (3.11%)
<b>Level of Education, n (%)</b>			
< HS	717 (37.04%)	1,473 (27.76%)	2,190 (30.24%)
HS/GED	639 (33.01%)	1,378 (25.97%)	2,017 (27.85%)
College	580 (29.96%)	2,455 (46.27%)	3,035 (41.91%)
<b>Alcohol</b>			
≤1 Drink/Day	334 (17.25%)	1,475 (27.28%)	1,809 (24.98%)
1-2 Drinks/Day	473 (24.43%)	1,419 (26.74%)	1,892 (26.13%)

<b>Table of Demographics by fortification period</b>			
	<b>Fortification Period</b>		
	<b>Pre n = 1,936</b>	<b>Post n = 5,306</b>	<b>Total n = 7,242</b>
<b>3+ Drinks/day</b>	1,129 (58.32%)	2,412 (45.46%)	3,541 (48.90%)
<b>Smoking</b>			
<b>Smoker</b>	1,105 (57.08%)	2,625 (49.47%)	3,730 (52.51%)
<b>Non-Smoker</b>	831 (42.92%)	2,681 (50.53%)	3,512 (48.49%)
<b>Masking Effect<sup>1</sup>, n (%)</b>			
<b>Present</b>	35 (1.81%) (2.05%) <sup>2</sup>	129 (2.43%) (2.32%) <sup>2</sup>	164 (2.26%) (2.24%) <sup>2</sup>
<b>Not present</b>	1,901 (98.19%) (97.95%) <sup>2</sup>	5,177 (97.57%) (97.68%) <sup>2</sup>	7,078 (97.74%) (97.76%) <sup>2</sup>

<sup>1</sup> Masking effect defined as having Serum B-12 <148 pmol/L with MCV <98 fL. All others that did not meet this criteria were defined as “no masking effect present.”

<sup>2</sup> Percent of weighted sample.

The distribution of each variable was analyzed using the Kolmogorov-Smirnov (KS) test for normality. This test was chosen over the Shapiro Wilks test because the sample size was larger than 2,000. Due to outliers and significant tails, the distributions for serum folate, B-12, MCV, and creatinine, as well as age, and PIR were not normally distributed as indicated by p-values <0.05 for each variable, and thus required non-parametric statistical test for analysis. Results from the normality test can be found in **Appendix A**. Due to the skewed distribution of the serum lab values, adjustment was required for reporting means. The geometric mean accounts for the effect of extreme outliers for the serum lab values. The geometric mean for pre-fortification serum folate was 11.68 nmol/L (95% CI, 11.36-12.00); serum B-12 was 335.00 pmol/L (95% CI, 328.32-341.18); serum creatinine was 1.07 mg/dL (95% CI, 1.06-1.08); and the

geometric mean of pre-fortification MCV was 89.62 fL (95% CI, 89.38-89.86).

Geometric means for serum concentrations in the post-fortification era are as follows:

folate 26.77 nmol/L (95% CI, 26.40-27.16), B-12 was 334.80 pmol/L (95% CI, 330.63-339.03), creatinine was 0.84 mg/dL (95% CI, 0.84-0.85), and MCV was 91.07 fL (95% CI, 90.92-91.22).

A significant difference in the distribution of age was seen between the two groups, as the average age of the participants increased in the post-fortification period (KS  $D = 0.16$ ,  $P < 0.001$ ). PIR also saw a significant shift in distribution, as it increased in the post-fortification group ( $D = 0.10$ ,  $P < 0.0001$ ). Serum folate levels significantly increased in the post-fortification period ( $D = 0.57$ ,  $P < 0.0001$ ), while no significant difference was found in the distribution of serum B-12 concentrations between the two groups ( $D = 0.02$ ,  $P < 0.4374$ ). Additionally, a decrease was observed in serum creatinine concentration in the post-fortification group, creating a significant difference in the distribution of serum creatinine levels ( $D = 0.44$ ,  $P < 0.0001$ ). A shift in MCV was also observed, as MCV was significantly different between the pre and post-fortification groups ( $D = 0.14$ ,  $P < 0.0001$ ). Education levels also changed significantly ( $D = 0.16$ ,  $P < 0.0001$ ) between the two periods, as the proportion of individuals that did not graduate high school fell and the proportion of those who graduated college rose in the post-fortification period. Furthermore, smoking and alcohol consumption both saw significant differences in proportions of responses between groups. More individuals reported smoking in the post-fortification era ( $D = 0.08$ ,  $P < 0.0001$ ). Alcohol consumption shifted down as the proportion of those reporting  $\leq 1$  drink/day increased from the pre-fortification period to the post-fortification period ( $D = 0.13$ ,  $P < 0.001$ ). Lastly, the

distribution of those identified with the markers for the masking effect was not statistically different between the pre and post-fortification groups ( $D=0.06$ ,  $P<0.7139$ ).

Results for the two-sample tests for each variable can be found in **Appendix B**.

Logistic regression analysis was performed using all variables where significant differences between pre and post-fortification periods were observed. All covariates were combined into a full model as predictors of the masking effect. The global model was significant (Wald  $X^2 = 90.49$ ,  $P<0.0001$ ), with the predictors of masking limited to race/ethnicity (Wald  $X^2 = 23.61$ ,  $P<0.0001$ ) and serum folate concentrations (Wald  $X^2 = 8.59$ ,  $P<0.0001$ ). The fortification period trended towards significance (Wald  $X^2 = 2.98$ ,  $P<0.085$ ), as did gender (Wald  $X^2 = 3.40$ ,  $P<0.065$ ). The analysis of effects for the global model is in **Table 2**.

**Table 2 Global Effect for the Post vs. Pre-fortification era**

<b>Global Analysis of Effects of Post vs. Pre</b>			
<b>Effect</b>	<b>DF</b>	<b>Wald Chi-Square</b>	<b>Pr &gt; ChiSq</b>
<b>PREPOST</b>	1	2.9758	0.0845
<b>Gender *</b>	1	3.4024	0.0651
<b>Stratified Age*</b>	3	2.7588	0.4303
<b>Age</b>	1	0.5630	0.4530
<b>Race/Ethnicity *</b>	3	23.6102	<.0001
<b>PIR *</b>	3	4.7567	0.1905
<b>Serum folate *</b>	1	8.5851	0.0034
<b>MCV</b>	1	17.2122	<.0001
<b>Education</b>	2	3.4855	0.1750
<b>Alcohol</b>	2	0.6321	0.7290

\* Sub-group analysis indicated significant risk increase of the masking effect for men, individuals 55 and older, non-Hispanic blacks, those in the third quartile for PIR, and low serum folate, defined as <6.3 nmol/L.

Subgroup analysis was performed for age, gender, race/ethnicity, education, PIR, serum folate, alcohol consumption, and smoking across fortification periods. Significant findings are reported in **TABLE 3**. Age was first stratified by twenty-year increments. No significant effect was seen with this level of stratification. Age was then stratified into those younger than 55 years and those equal to, or older than 55. For those in the post-fortification period, being older than 55 years of age was a significant predictor of displaying low B-12 with no macrocytosis (Wald  $X^2= 3.90$   $P<0.048$ ) compared to their pre-fortification counterparts. This corresponded with more than a 2-fold increase in the likelihood of displaying the masking effect in the post-fortification era than the pre-fortification period for this age group (OR= 2.18, 1.01-4.74). Being less than 55 years old was not a significant predictor of the masking effect in the post-fortification period.

**Table 3 Significant outcomes from the analysis of effects in the Post-fortification period versus the Pre-fortification period.**

<b><u>Analysis of Effects for Post vs. Pre</u></b>						
<b>Effect</b>	<b>DF</b>	<b>Wald Chi-Square</b>	<b>Pr &gt; ChiSq</b>	<b>Odds Ratio</b>	<b>95% Wald Confidence Limit</b>	
<b>Gender</b>						
Male	1	6.3075	0.0120	2.645	1.238-5.650	
<b>Race/Ethnicity</b>						
NH black	1	4.1069	0.0427	3.115	1.038-9.350	
<b>Stratified Age</b>						
>55 (y)	1	3.9044	0.0482	2.183	1.006-4.737	
<b>Serum Folate<sup>2</sup></b>						
<6.8 nmol/L	1	13.6459	0.0002	35.885	5.371-239.773	
<b>Smoking Status</b>						
Non-smoker	1	11.4197	0.0007	4.631	1.904-11.267	
<b>PIR by Quartile</b>						

<b><u>Analysis of Effects for Post vs. Pre</u></b>						
<b>Effect</b>	<b>DF</b>	<b>Wald Chi-Square</b>	<b>Pr &gt; ChiSq</b>	<b>Odds Ratio</b>	<b>95%Wald Confidence Limit</b>	
<b>Q3</b>	1	3.9498	0.0469	3.216	1.016-10.117	

<sup>1</sup> Serum folate <6.8 nmol/L was one of four stratification levels. When re-stratified to concentrations above and below 6.8 nmol/L, no effect was seen for serum folate and masking in the post-fortification era.

<sup>2</sup> The third quartile for PIR was one of four stratification levels. When re-stratified to scores at and above 4.22, and those below, no effect was seen for PIR and the masking effect in the post fortification era.

Gender had a significant effect in the post-fortification era, as being male proved to be a significant predictor of the making effect (Wald  $X^2 = 88.14$ ,  $P < 0.0001$ ). When compared to men in the pre-fortification group, men in the post-fortification era had a nearly two-fold increase in the likelihood of being B-12 deficient with normal MCV (OR= 2.65, 1.24-5.65). No significant effect was found for women (Wald  $X^2 = 0.31$ ,  $P < 0.5774$ ). Race/ethnicity, specifically being non-Hispanic blacks, had a significant effect on being B-12 deficient without macrocytosis in the post-fortification period (Wald  $X^2 = 4.12$ ,  $P < 0.0427$ ). Non-Hispanic blacks in the post-fortification era saw a three-fold increase in the odds of displaying the masking effect (OR=3.12, 1.04-9.35) compared to their NHANES III counterparts. No other race/ethnicity proved to be a significant predictor.

No significance was observed for the effect of education on the masking effect in the pre versus post-fortification groups. Nor was education significant when re-stratified to those who did not graduate high school versus those who did across fortification periods. Smoking status, however, was significant at predicting of the potential masking. Being a non-smoker in the post-fortification era proved to be a significant predictor of the masking effect, increasing the odds of displaying the effect nearly 5-fold (OR= 4.63,

1.90-11.27). The act of smoking had no predictive abilities for the masking effect. Alcohol consumption had no effect on low B-12 without macrocytosis.

An effect for PIR was seen when stratified by quartiles. Only the third quartile was a significant predictor of the masking effect in the post-fortification period (Wald  $X^2= 3.95$ ,  $P<0.047$ ). For those in the third quartile of PIR, which consisted of a range of 4.22-4.9, increased likelihood of being B-12 deficient with normal MCV existed in the post fortification period compared to those in the pre-fortification period (OR= 3.22, 1.016-10.177). All other quartiles were not significant predictors of the masking effect in the post-fortification period. No significant effect was seen when those at and above a PIR of 4.22 were compared to those below 4.22 for the pre and post-fortification time periods. When stratified to above, and below or equal to the median (2.38), no significant predictive effect was seen for PIR. Additionally, no effect was seen when stratified to above or below a PIR of 1.5, or 150% of the poverty line.

Serum folate was a significant predictor of the masking effect in the global model. When stratified using the concentrations established by the World Health Organization (WHO) for deficiency, insufficiency, normal, and elevated levels, a significant effect was seen for serum folate less than 6.2 nmol/L, the cutoff for deficiency for the WHO (Wald  $X^2= 8.59$ ,  $P<0.0034$ ). When re-stratified to those above and those below 6.2 nmol/L, no effect was seen. No other level of serum folate was a significant predictor of the masking effect in the post-fortification era. Serum creatinine was not significant in the global model, nor when stratified to those above and below 1.1 mg/L, the upper cutoff for normal creatinine levels in women, nor when stratified above or below 1.3 mg/L, the upper cut off for men.



## CHAPTER V

### DISCUSSION AND CONCLUSION

#### Association between the masking effect, fortification, and confounders

This was the first epidemiological investigation into the direct association the masking effect of B-12 deficiency between the pre- and post folic acid fortification populations using NHANES data. Serum folate levels have significantly increased in the post-fortification period compared to the pre-fortification group, in accord with current literature.<sup>8,10</sup> The United States Institute of Medicine (IOM) reviewed potential adverse effects of increased intake of folic acid prior to the fortification mandate in January 1998. From this investigation the IOM found that the only potential risk associated with increased serum folate was the potential masking effect of vitamin B-12 deficiency. Despite the risk of masking B-12 deficiency, folic acid fortification was mandated for processed grains in the United States effective January 1<sup>st</sup> 1998. The result of this program reduced neural tube defects in the United States by as much as 19%.<sup>4</sup> Other fortification programs have seen similar results, including in Chile which saw a decrease in incidence of NTDs by 50%, from 17.1 to 8.6/100,000 live births.<sup>29</sup>

The prevalence of those with serum B-12 <148 pmol/L was 2.49%, and prevalence of those who met the criteria for the presence of the masking effect was 2.26% in the combined NHANES sample population. The proportion of those who met criteria for the

masking effect increased from 1.81% in the pre-fortification period to 2.43% in the post-fortification era. The weighted proportions also saw an increase in the post fortification era, from 2.05% to 2.32%, however this was not a statistically significant increase ( $KSa = 0.87$ ,  $P < 0.4374$ ). Therefore, we reject the hypothesis that the prevalence of the masking effect has significantly increased in the post-fortification period. Despite this, the weighted sub-sample population who met the criteria for the masking effect was representative of over 1.4 million individuals in the post-fortification period at risk of the potential deleterious effects associated with undiagnosed vitamin B-12 deficiency. While this is not a definitive prevalence rate it is indicative of the need for further study, as this is a substantial estimated population at risk.

The main findings of this study, no significant increase in the masking effect in the post-fortification period, contradict that of Wyckoff and Ganji (2007), which found a significant increase in the proportion of those with low serum B-12 without macrocytosis. However, difference in selection and size of the study populations, cut-off value for B-12 deficiency and between the two studies could account for this difference in outcome. In their study, Wyckoff and Ganji compiled data from patients at Rush Medical University Hospital Despite versus this study's use of NHANES population-wide random, stratified clustered sampling. Additionally, the cutoff value for low serum B-12 in their study was set at a more liberal 258 pmol/L, versus the more conservative 148 pmol/L used in this study.

Mills et al (2003), in their investigation into the temporal effect of folic acid fortification, found that the proportion of those with B-12 deficiency without macrocytosis did not significantly change between the pre and post-fortification eras,

similar to the findings of this study. However, the sample populations differ between the two studies in selection methods, as the NHANES is a randomized stratified cluster study, whereas Mills et al compiled data from the Veteran's Affairs Medical Center in Washington, DC. Furthermore, the cut-off value to denote B-12 deficiency in the Mills study was 258 pmol/L versus the more conservative 148 pmol/L used in this study. In contradiction to Mills et al (2011), which found none of their sample population displayed macrocytosis, 16 individuals were identified as displaying macrocytosis in this study. When low serum folate levels were taken into account, 14 individuals remained, indicating true B-12 deficiency. Applying the sample weight indicated that those 14 individuals were representative of nearly 200,000 in the general population.

The final sample population contained a higher proportion of men than women than one would expect to see in the general population. The youngest age included in analysis was 17 years, despite inclusion of children in the original dataset. This is due primarily to the missing data for alcohol consumption for youth under the legal drinking age in the continuous NHANES survey series, as the question was reserved for those of legal drinking age. Therefore, those below the drinking age in the continuous NHANES were lost to analysis. The NHANES survey design intentionally over-samples certain demographics, however cluster, stratum and sample weight variables were created for each individual to account for this variation. Hispanic/Mexican Americans were over-represented in the final sample population when compared to the general population, comprising 23.6% of the combined NHANES sample population.

The shift in education status indicates that a larger proportion of individuals attending college in the post-fortification era. Interestingly, smokers comprised nearly

49% of the total sample population, which is substantially higher than is seen in the general population estimates reported by the Centers for Disease Control and Prevention (CDC).<sup>30</sup> Alcohol consumption was heavily skewed towards those who consumed more than three drinks per day. This could be due to recoding the variables between the NHANES III and continuous NHANES series, which differed in the questions related to alcohol consumption. The decrease in MCV levels was consistent with the findings of Wyckoff and Ganji (2007) from the pre- to post-fortification period. Although significantly different, both average MCV levels were within a clinically normal range. While in agreement with Wyckoff and Ganji, the lowering of MCV levels in the post-fortification period contradicts the finding of Hirsh et al (2002) who found an increase in MCV after mandatory folic acid fortification of flour in Chile. Lastly serum creatinine levels were significantly lower in the post-fortification period, potentially indicating better renal function in the post-fortification group.

#### Likelihood for the masking effect for the pre and post-fortification era

The association of low serum B-12 without macrocytosis and folic acid fortification period was significant gender, race/ethnicity, age, and PIR. More specifically, and taken as individual predictors, males, non-Hispanic blacks, those who were older than 55, and those in the third quartile for PIR in the post-fortification population had a higher likelihood of being B-12 deficient with normal MCV compared to their pre-fortification counterpart. The findings for the effect of race/ethnicity and age are in agreement with those of Wyckoff and Ganji (2007). When age was stratified to those above and those at or below 60, neither group proved to be a significant predictor of the masking effect. This is discordant with Mills (2003) who found that those aged

<65 years were more likely to be B-12 deficient without macrocytosis. Men were more likely to meet the criteria for the masking effect in the post fortification period compared to men in the pre-fortification period, a similar finding to Wyckoff and Ganji (2007). However, the same effect was not seen in women.

Folic acid was a strong global predictor of the masking effect in the post-fortification period. However when stratified by serum concentrations defined by the WHO as deficient, insufficient, replete, and elevated, the only association between fortification periods was seen for folate deficient individuals. When re-stratified to those below the deficient cut off and those above, no association across fortification period was seen. No effect was seen for the highest strata for serum folate, which is in accord with MacFarlane et al (2011) who found those with elevated serum folate were more likely to be B-12 replete compared to those with serum folate within the normal range.<sup>26</sup> As for potential confounding variables, education and alcohol consumption were not found to be predictors, and did not contribute to the likelihood of displaying the masking effect in the post-fortification period. However, smoking status was a significant predictor. Non-smokers saw an increased risk of the masking effect in the post-fortification period when compared to non-smokers in the pre-fortification era. This effect was not seen in smokers. However, this finding should not be taken as a potential protective effect of smoking, as cigarette use has been shown to increase the risk of numerous medical issues.

For those in the third quartile for PIR, the odds of meeting the criteria for the masking effect significantly increased in the post-fortification period versus the pre-fortification period. Because race/ethnicity has been implicated in increasing the odds of

displaying the masking effect in several studies, further investigation into the role race/ethnicity plays in B-12 status is warranted.

### Strengths and Limitations

One of the strengths of this study is that it incorporated a large sample size (n=7,242) that provided power to the statistical analysis performed. SAS 9.3 software is a newer version that allows for analysis of complex survey designs, like the NHANES. This allowed for the adjustment of the analysis to account for the clustering and stratification of the survey design and the intentional over-sampling of certain demographics that occur within the NHANES surveys. Another strength of this study was that it was the first epidemiological investigation exclusively into the masking effect of folic acid using serum folate, and vitamin B-12 concentrations in conjunction with MCV levels as a measure of macrocytic anemia using population-wide data.

Limitations of this study include the design of NHANES itself. As a cross-sectional study, analysis of NHANES data cannot produce cause-and-effect relationships. SAS 9.3 is newer version of the software, however there is a limited scope to capabilities of complex analysis. Although this software is powerful enough to analyze the data, NCHS recommends the use of SUDANN for performing logistic regression with NHANES surveys data. SUDANN produces the Wald-F statistic, Satterthwaite adjusted-F, and Satterthwaite  $X^2$ , whereas SAS only produces the Wald F statistic. However, the NHANES analytic guidelines do not make a recommendation as to which test is the best, only that the Satterthwaite adjusted F statistic is the more conservative test and, therefore, less likely to reject the null than is the Wald F alone. Additionally, supplementation of folic acid or vitamin B-12 was not accounted for in the analysis, which likely resulted in

the extreme outliers serum concentrations for folate and B-12. Although care was taken, coding errors could have occurred when reformatting, scrubbing, or creating new variables with the data, which would add error into the analysis.

### Conclusions

This study investigated the effect of folic acid fortification on the risk of presenting with vitamin B-12 deficiency without macrocytosis. Although the proportion of those with vitamin B-12 deficiency without macrocytosis increased in the post-fortification era, the increase was not statistically significant. A significant increase in serum folate concentration has occurred in the United States since the introduction of mandatory folic acid in processed grains in 1998. However, serum B-12 concentrations saw no significant change between pre and post-fortification periods. Compared to the pre-fortification period, in the post-fortification era the likelihood of being B-12 deficient without macrocytosis was significantly increased for men, non-Hispanic blacks, those over the age of 55, and for those in the third quartile for PIR. But the effect for PIR was only seen when stratified by quartiles. No effect for PIR was seen when stratified by those above and below the third quartile. Although serum folate levels below 6.3 nmol/L were found to be significantly associated with the masking effect in the post-fortification era, further analysis refuted this evidence. Elevated serum folate levels were not a predictor of the masking effect in the post fortification era. Further investigations are warranted into the masking effect, especially as pertains to the impact race/ethnicity may have.

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## APPENDIX A

### Tests for Normality

<b>Tests for Normality</b>					
<b>Variable</b>	<u>Pre-fortification</u>			<u>Post-fortification</u>	
	<b>Test</b>	<b>Statistic</b>	<b>p Value</b>	<b>Statistic</b>	<b>p Value</b>
	<b>Kolmogorov -Smirnov</b>	<b>D</b>	<b>Pr &gt; D</b>	<b>D</b>	<b>Pr &gt; D</b>
<b>Age</b>		0.102814	<0.0100	0.060172	<0.0100
<b>PIR</b>		0.102649	<0.0100	0.120898	<0.0100
<b>Serum B-12</b>		0.427912	<0.0100	0.419876	<0.0100
<b>Serum Folate</b>		0.427912	<0.0100	0.419876	<0.0100
<b>Creatinine</b>		0.146886	<0.0100	0.197073	<0.0100
<b>MCV</b>		0.046294	<0.0100	0.062152	<0.0100

<b>Moments for Variables</b>								
<b>Variable</b>	<u>Pre-fortification</u>				<u>Post-fortification</u>			
	<b>Mean</b>	<b>Median</b>	<b>IQR</b>	<b>Kurtosis</b>	<b>Mean</b>	<b>Median</b>	<b>IQR</b>	<b>Kurtosis</b>
	<b>(sd)</b>				<b>(sd)</b>			
<b>Age</b>	43.43 (16.74)	40.0	26.00	-0.5933	48.21 (17.71)	47.00	29.00	-0.9559
<b>PIR</b>	2.39 (1.56)	2.02	2.65	-1.1059	2.73 (1.62)	2.53	3.16	-1.3980
<b>Serum B-12</b>	425.67 (1898)	327.21	173.76	1241.39	407.46 (1612)	332.10	183.0	3967.84
<b>Serum Folate</b>	14.43 (12.12)	10.90	9.50	48.3557	31.03 (20.15)	26.30	19.10	42.9050
<b>Serum Creatinine</b>	1.09 (0.22)	1.10	0.20	20.3800	0.88 (0.39)	0.80	0.30	328.1877
<b>MCV</b>	89.78 (5.26)	89.95	6.20	2.3079	91.23 (5.29)	91.40	5.90	3.9442

**APPENDIX B**

Two-sample Tests

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**Kolmogorov-Smirnov Test for Serum Folate  
Classified by Variable PREPOST**

<b>PREPOST</b>	<b>N</b>	<b>EDF at Maximum</b>	<b>Deviation from Mean at Maximum</b>
<b>Pre-fortification</b>	1936	0.697314	18.384663
<b>Post-fortification</b>	5306	0.127026	-11.105157
<b>Total</b>	7242	0.279481	

**Maximum Deviation Occurred at Observation  
1818**

**Value of FOPSI at Maximum = 15.0**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

<b>KS</b>	0.252390	<b>D</b>	0.570288
<b>KSa</b>	21.478369	<b>Pr &gt; KSa</b>	<.0001

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**Kolmogorov-Smirnov Test for Serum B-12  
Classified by Variable PREPOST**

<b>PREPOST</b>	<b>N</b>	<b>EDF at Maximum</b>	<b>Deviation from Mean at Maximum</b>
<b>Pre-fortification</b>	1936	0.104339	-0.743529
<b>Post-fortification</b>	5306	0.127403	0.449125
<b>Total</b>	7242	0.121237	

**Maximum Deviation Occurred at Observation  
3686**

**Value of VBPSI at Maximum = 206.640**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

<b>KS</b>	0.010207	<b>D</b>	0.023064
<b>KSa</b>	0.868647	<b>Pr &gt; KSa</b>	0.4374

.....  
The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for Serum Creatinine  
Classified by Variable PREPOST**

<b>PREPOST</b>	<b>N</b>	<b>EDF at Maximum</b>	<b>Deviation from Mean at Maximum</b>
<b>0</b>	1936	0.244318	-14.026581
<b>1</b>	5306	0.679420	8.472681
<b>Total</b>	7242	0.563104	

**Maximum Deviation Occurred at Observation  
3598**

**Value of CEP at Maximum = 0.90**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

<b>KS</b>	0.192561	<b>D</b>	0.435101
<b>KSa</b>	16.386925	<b>Pr &gt; KSa</b>	<.0001

.....  
The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for PIR  
Classified by Variable PREPOST**

<b>PREPOST</b>	<b>N</b>	<b>EDF at Maximum</b>	<b>Deviation from Mean at Maximum</b>
<b>0</b>	1936	0.729855	3.302811
<b>1</b>	5306	0.627403	-1.995045
<b>Total</b>	7242	0.654791	

**Maximum Deviation Occurred at Observation  
1378**

**Value of DMPPIR at Maximum = 3.3520**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

<b>KS</b>	0.045342	<b>D</b>	0.102452
<b>KSa</b>	3.858596	<b>Pr &gt; KSa</b>	<.0001

.....  
The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for AGE  
Classified by Variable PREPOST**

PREPOST	N	EDF at Maximum	Deviation from Mean at Maximum
0	1936	0.612087	5.022953
1	5306	0.456276	-3.034088
<b>Total</b>	7242	0.497929	

**Maximum Deviation Occurred at Observation  
3643**

**Value of HSAGEIR at Maximum = 44.0**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

<b>KS</b>	0.068957	<b>D</b>	0.155811
<b>KSa</b>	5.868198	<b>Pr &gt; KSa</b>	<.0001

The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for MCV  
Classified by Variable PREPOST**

PREPOST	N	EDF at Maximum	Deviation from Mean at Maximum
0	1936	0.605888	4.610486
1	5306	0.462872	-2.784939
<b>Total</b>	7242	0.501105	

**Maximum Deviation Occurred at Observation  
1918**

**Value of MVPSI at Maximum = 91.050**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

<b>KS</b>	0.063294	<b>D</b>	0.143016
<b>KSa</b>	5.386322	<b>Pr &gt; KSa</b>	<.0001

The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for Level of Education  
Classified by Variable PREPOST**

PREPOST	N	EDF at Maximum	Deviation from Mean at Maximum
Pre-fortification	1936	0.700413	5.257839
Post-fortification	5306	0.537316	-3.175970
Total	7242	0.580917	

Maximum Deviation Occurred at Observation  
3554

Value of N3\_Edu at Maximum = 2.0

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

KS	0.072181	D	0.163097
KSa	6.142610	Pr > KSa	<.0001

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The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for Gender  
Classified by Variable PREPOST**

PREPOST	N	EDF at Maximum	Deviation from Mean at Maximum
Pre-fortification	1936	0.625000	0.797432
Post-fortification	5306	0.600264	-0.481684
Total	7242	0.606877	

Maximum Deviation Occurred at Observation  
3601

Value of HSSEX at Maximum = 1.0

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

KS	0.010947	D	0.024736
KSa	0.931621	Pr > KSa	0.3506

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The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for Stratified Age  
Classified by Variable PREPOST**

PREPOST	N	EDF at Maximum	Deviation from Mean at Maximum
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**Kolmogorov-Smirnov Test for Stratified Age  
Classified by Variable PREPOST**

PREPOST	N	EDF at Maximum	Deviation from Mean at Maximum
Pre-fortification	1936	0.487087	4.067554
Post-fortification	5306	0.360912	-2.456984
Total	7242	0.394642	

**Maximum Deviation Occurred at Observation  
3620**

**Value of Age\_strat at Maximum = 2.0**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

KS	0.055841	D	0.126175
KSa	4.752028	Pr > KSa	<.0001

The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for Alcohol  
Classified by Variable PREPOST**

PREPOST	N	EDF at Maximum	Deviation from Mean at Maximum
Pre-fortification	1936	0.416839	-4.145144
Post-fortification	5306	0.545420	2.503852
Total	7242	0.511047	

**Maximum Deviation Occurred at Observation  
3749**

**Value of Alcohol at Maximum = 2.0**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

KS	0.056906	D	0.128581
KSa	4.842675	Pr > KSa	<.0001

The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for Smoke  
Classified by Variable PREPOST**



<b>PREPOST</b>	<b>N</b>	<b>EDF at Maximum</b>	<b>Deviation from Mean at Maximum</b>
<b>0</b>	1936	0.570764	2.451388
<b>1</b>	5306	0.494723	-1.480748
<b>Total</b>	7242	0.515051	

**Maximum Deviation Occurred at Observation  
3644**

**Value of smoke at Maximum = 1.0**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

<b>KS</b>	0.033653	<b>D</b>	0.076042
<b>KSa</b>	2.863899	<b>Pr &gt; KSa</b>	<.0001

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The NPAR1WAY Procedure

**Kolmogorov-Smirnov Test for Variable Masking  
Classified by Variable PREPOST**

<b>PREPOST</b>	<b>N</b>	<b>EDF at Maximum</b>	<b>Deviation from Mean at Maximum</b>
<b>Pre-fortification</b>	1936	0.018079	-0.200955
<b>Post-fortification</b>	5306	0.024312	0.121386
<b>Total</b>	7242	0.022646	

**Maximum Deviation Occurred at Observation  
3554**

**Value of Masking at Maximum = 1.0**

**Kolmogorov-Smirnov Two-Sample Test  
(Asymptotic)**

<b>KS</b>	0.002759	<b>D</b>	0.006234
<b>KSa</b>	0.234771	<b>Pr &gt; KSa</b>	1.0000