LEVELS OF RED BLOOD CELL FOLIC ACID AND A HISTORY OF PEPTIC

ULCER DISEASE

by

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

Computer-assisted personal interview	CAPI
Centers for Disease Control and Prevention	CDC
Composite International Diagnostic Interview	CIDI
Delta Cell	D cell
Deoxyribonucleic acid	DNA
Enzyme linked Immunosorbent Assay	ELISA
Esophagogastroduodenoscopy	EGD
Food and Drug Administration	FDA
General purpose statistical software package	STATA
Helicobacter *pylori* is equivalent to	H. *pylori*
Institutional Review Board	IRB
National Center for Health Statistics	NCHS
National Health and Nutrition Examination Survey	NHANES
National Institute of Health	NIH
Non steroidal anti inflammatory drug	NSAID
Red Blood Cell	RBC
Statistical Analysis System	SAS
United States of America	USA
United States Department of Agriculture	USDA
United States Environmental Protection Agency	USEPA
United Kingdom	UK

ACKNOWLEDGEMENTS

ABSTRACT

Background: In the United States about half a million people develop peptic ulcer each year, and the health care costs of this disease amounts to an estimated value of $10 billion annually. To date, there have been no studies evaluating the association between folic acid consumption or folic acid levels, and reduction in peptic ulcers. It might be possible that the mechanism of action of folic acid on amino acid and DNA synthesis might help cells regenerate and act protectively against peptic ulcers.

Objective: To evaluate the association between the level of red blood cell folic acid and a history of peptic ulcers using the National Health and Nutrition Examination Survey (NHANES) database from the years 1999-2000.

Method: The data for this study were from the NHANES 1999-2000 online public use database. All statistical analyses accounted the complex sample survey design of the NHANES sampling weights. For data analysis we used pearson's chi-square for categorical variables and t-test for continuous variables. Confounders were selected and added one at a time to the model. Those that changed the association between RBC folic acid and lifetime peptic ulcers by 10% or more were considered as confounders. We also conducted a weighted Wald test for interactions between folic acid and gender, and folic acid and *H.pylori*. Our outcome variable was defined as peptic ulcer within a lifetime in the (1)/(0) format, while our main exposure was defined as RBC folic acid level. For this purpose we considered a multivariate logistic regression model analysis with a cutoff for confounder inclusion of $p < 0.25$. The confounder variables considered

necessary after their evaluation for possible inclusion in the final multivariate logistic regression model were gender, marital status, ethnicity, age, level of education, smoking exposure, stomach or intestinal illness, income, and veteran or military status. All data analyses were conducted using STATA 11.

Results: The association between RBC folic acid levels and a lifetime of peptic ulcers was not statistically significant in either univariate or multivariate logistic regression analyses. Our model showed that as compared to the baseline range of 0ng/ml to 227ng/ml, the odds of ever having a lifetime peptic ulcers remain mostly the same at the 228ng/ml to 301ng/ml range (OR= 1.00; 95% CI: 0.54-1.87; p=0.98), the odds of developing a lifetime peptic ulcers decreases at the 302ng/ml to 628ng/ml range (OR=0.79; 95% CI: 0.40-1.58; p=0.48), and the odds of ever having a lifetime peptic ulcers also decreases above 629ng/ml (OR=0.82; 95% CI: 0.44-1.55; p=0.53). However, this dose response effect of decreasing odds of lifetime peptic ulcers with increasing RBC folic acid was not statistically significant (p= 0.393; trend test).

Discussion: This study failed to find a significant association between RBC folic acid levels and lifetime peptic ulcers. The data suggests a decrease in lifetime peptic ulcers, as the blood levels of RBC folic acid increase; however, this trend is not supported through statistical significance. Although this is the first study evaluating this association, additional studies are needed to determine a true association between blood levels of RBC folic acid and a reduction in peptic ulcers. Patients, providers, and researchers will benefit from the information produced by this study.

Conclusions: Statistically, it is difficult to determine that a dose response association truly exists between RBC folic acid levels and lifetime peptic ulcers. Further studies might validate the dose response trend found in this study. It is biologically possible that an association exists because folic acid helps with the synthesis of DNA and amino acids. Therefore, it is plausible to think that folic acid helps restore cells affected through ulcerations in the gastrointestinal tract, thus decreasing the likelihood of peptic ulcers.

INTRODUCTION

In the United States, approximately half a million people develop a peptic ulcer each year, and the health care costs of this disease amounts to an estimated cost of $10 billion annually[1]. No studies have been conducted to evaluate the association between folic acid consumption or folic acid levels, and peptic ulcers. An examination of this association could be significant not just in treating patients who suffer from peptic ulcers, but for those who also take non steroidal anti inflammatory drugs (NSAIDs), since these medications are known to cause adverse gastrointestinal reactions. This study is a cross sectional study evaluating association between the level of red blood cell (RBC) folic acid and a history of peptic ulcers in the United States. Current studies show that trends in peptic ulcers not related to *H. pylori* and NSAIDs consumption will remain stable, whereas those related to *H. pylori* will decrease. A study conducted in the U.S. reported that 47% of white patients and 22% of non white patients with ulcers were not using NSAIDs and tested negative for *H. pylori*[2]. Therefore, the non H. pylori and non NSAIDs related peptic ulcers will have important implications in future clinical management, as *H. pylori* will not be a reasonable predictive parameter to determine peptic ulcers. Instead, future patients will need maintenance acid suppressive therapy to prevent ulcer recurrence.

To avoid such chronic health condition and treatment costs, alternative inexpensive and non invasive solutions should be developed. A viable and inexpensive alternative might be consumption of folic acid. A study demonstrated that patients deficient in vitamin B_{12}, folic acid, and iron who followed a replacement therapy showed a recovery of oral ulceration, in

particular recovery was greater for those who increased their folic acid and B_{12} levels to normal[3]. Therefore, due to similarities in epithelial tissue, folic acid may be useful in preventing peptic ulcer recurrence or ulcers overall if taken before the onset of this gastrointestinal condition to avoid acid suppressive therapies. Also, the mechanism of action of folic acid on amino acid and DNA synthesis might help cells regenerate and act protectively against the NSAIDs mechanism of action on COX inhibition that causes the gastric irritation side effect.

RESEARCH QUESTION/HYPOTHESIS

Among NHANES participants in 1999-2000, we hypothesize that an association exists between RBC folic acid and lifetime peptic ulcers in ages 20 to 80.

SPECIFIC AIMS

1. Evaluate the association between RBC folic acid and lifetime peptic ulcers.

2. Evaluate the association between NSAIDs use and a lifetime peptic ulcers.

3. Evaluate whether NSAIDs use modifies the association between RBC folic acid and a lifetime of peptic ulcers.

4. Evaluate whether the presence of *H.pylori* modifies the association between RBC folic acid and a lifetime of peptic ulcers.

5. Evaluate weather gender modifies the association between RBC folic acid and lifetime peptic ulcers.

BACKGROUND

<u>Folic acid source and importance in the body</u>

Folate is a B vitamin that is found in a myriad of foods such as dark and leafy green vegetables, citrus fruits, yeast, beans, eggs, chicken liver and milk[4]. It is also important for biosynthesis, DNA replication, methyl group supply, and overall cell growth and repair[5]. Folate acts mainly with folate-dependent coenzymes, which work in accepting and donating one carbon units. The folate-coenzyme combination forms an enzyme that is critical for DNA synthesis, cell differentiation, and metabolism of amino acids; these processes occur in the cytosol, nucleus, and mitochondria within the cell. Folate is therefore important in cell division during the first weeks of pregnancy, and without it the fetus would not develop properly. It is also essential in the synthesis of red blood cells and damaged cell repair. In addition, folate works together with vitamins B12 and B6 in the metabolism of the essential amino acid methionine[6]. Folate deficiencies lead to megaloblastic anemia and ultimately to severe neurological problems[4].

A mandatory fortification of folic acid in cereal products was introduced in 1998 by the U.S. government. The intention was to decrease the risk of neural tube defects. The Food and Drug Administration required that fortification be enriched with a folic acid concentration of

140µg/100g of cereal grain. A study using NHANES data showed that between 1988-1994 and 1999-2000 the levels of serum and RBC folic acid in the U.S. increased from 12.0 (11.5, 12.5)nmol/L to 29.7 (27.9, 31.6) nmol/L, and 398 (389, 408) nmol/L to 636 (606, 668) nmol/L respectively. The commonly defined cutoff for serum folate levels is less than 6.8 nmol/L, and for RBC folic acid is less than 317 nmol/L. The prevalence of low serum folate concentration decreased from 16% to 0.5%, and the RBC folate concentration decreased from 31% to 3% [7].

Serum and red blood cell (RBC) folate units of measure in the blood

To assess folate status, measurements of serum and RBC folate are conducted in a lab by drawing blood samples from a patient and placing them in a chemiluminescent immunoassay. The serum folate level, however, is an indicator of recent folate intake. RBC folate level, on the other hand, is a good indicator of long term folate stores, and a value low in RBC folate is indicative of a continuous folate deficiency. The reference ranges are different for serum and RBC folate. The normal serum folate level is above 4.0ng/ml, indeterminate at 3.7-3.9ng/ml, and deficient below 3.7ng/ml. In the case of RBC folate, the normal level range is 187 to 645ng/ml, indeterminate range level is 149-186 ng/ml, and a deficient level is below 149ng/ml[4].

Folic acid synthesis of DNA and amino acid metabolism

Folic acid is composed of a pteridine connected to a p-aminobenzoic and L-glutamic acids (figure 1). It is known as pteroglytamic acid (PtGlu) because of the presence of pteridine and glutamic acid. However, folic acid was proposed as a name because of its abundance in leafy material[8]. This compound exists mostly in plant and animal tissue largely in the form of conjugates that have more than one glutamic acid[9].

Figure 1: Molecular structure of folic acid[10].
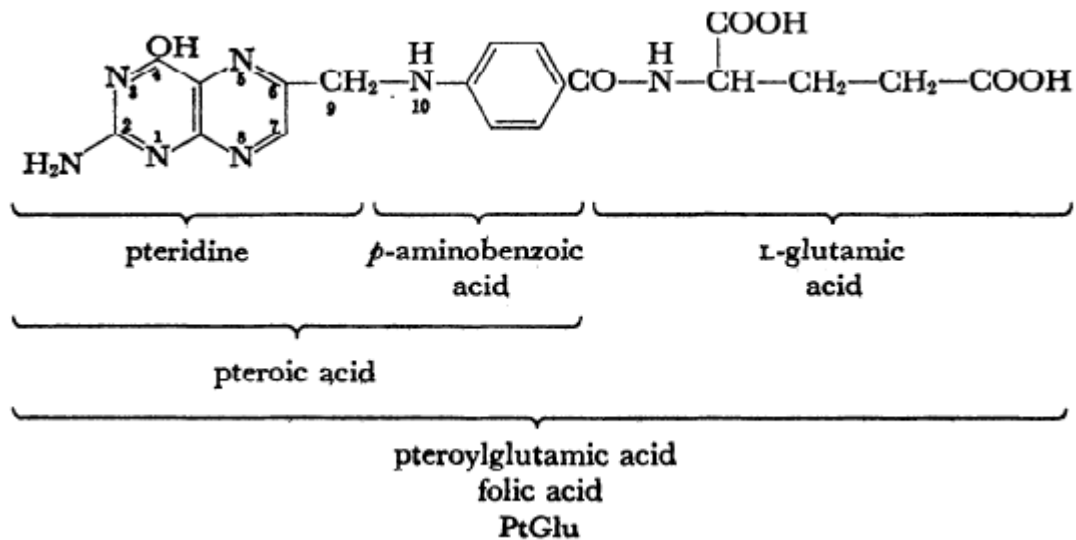
In the same manner as many other vitamins, folic acid is a precursor or a coenzyme that carries out an enzymatic reaction. Folate coenzymes are mostly concerned with the transfer of one-carbon units at the oxidation levels of formate, formaldehyde, methanol, and by

transforming these from one oxidation state to another. In addition, it is involved in the

synthesis of DNA and in the metabolism of amino acids. It is therefore linked in the synthesis of

purines and thymines[11,12,13], and folic acid acting as a coenzyme is involved in single-carbon

transformation reactions in purines, pyrimidines, serine, glycine, histidine, and methionine

(figure 2)[10].

Figure 2: Folate and nucleic acid metabolism on the right. Homocysteine metabolism used for synthesis of various aminoacids on the left[10].
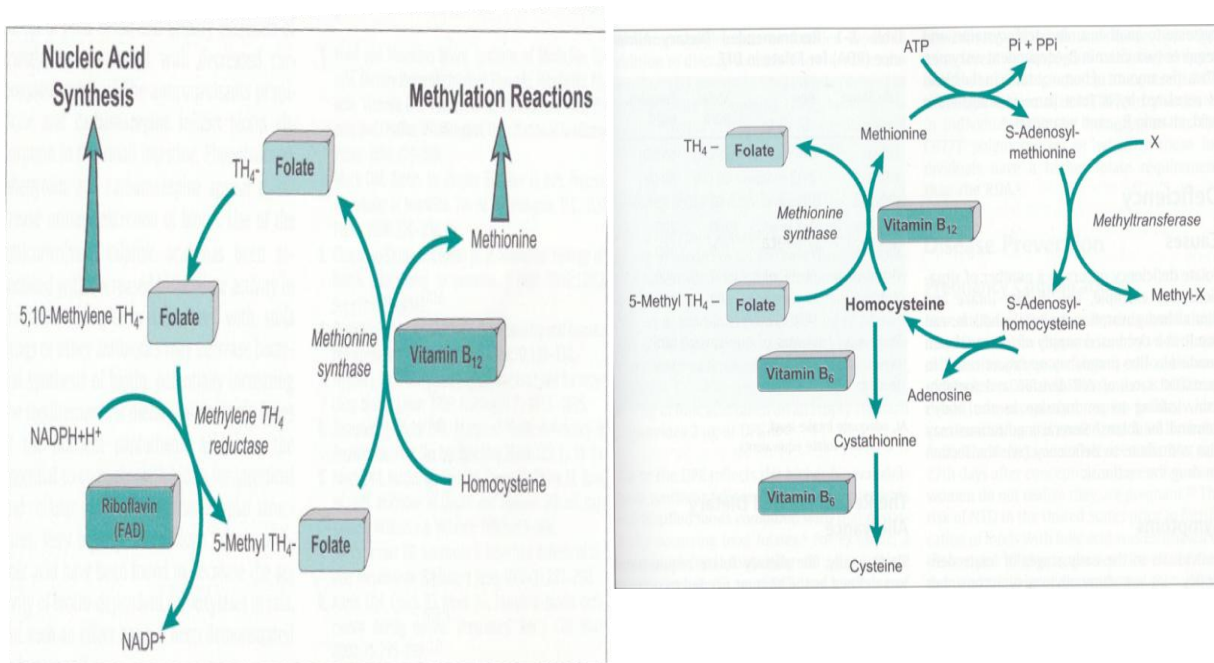
Peptic ulcer onset and prevalence in the USA and UK

A peptic ulcer is considered as a lesion on the tissue lining the stomach or duodenum. A peptic ulcer that occurs in the area of the stomach is known as a gastric ulcer. If the peptic ulcer is located in the duodenum, it is called a duodenal ulcer. Less commonly, lesions can occur in the lower esophagus, the distal duodenum, or the jejunum. People can have one or the other, or both at the same time. Most peptic ulcers occur due to Helicobacter *pylori*, a type of bacteria that causes an infection; however, the use of non steroidal anti inflammatory drugs (NSAIDs) is also associated with this condition and reported in many studies[15,16]. Smoking or drinking alcohol may worsen the status of a peptic ulcer and prevent healing. Other conditions that cause this gastrointestinal condition include hypersecretory states, such as Zolliger-Ellison syndrome, hiatal hernias, or ectopic gastric mucosa. Also, other comorbodities that might increase the risk of peptic ulcer are cytomegalovirus, tuberculosis, Crohn's disease, hepatic cirrhosis, chronic renal failure, sarcoidosis, and myeloproliferative disorders[16,17].

Common symptoms of peptic ulcers include a dull or burning pain that occurs when the stomach is empty, during the night, or between meals. The pain may last for minutes to hours, or come and go for several days or even weeks. In the case of duodenal ulcers the pain may be briefly relieved by eating foods; similarly, in the case of both duodenal and stomach ulcers the pain is relieved by taking antacids. [16].

Sonnenberg and colleagues reported the prevalence of peptic ulcers in the United States that were diagnosed by a physician. The prevalence of peptic ulcer within the last year at the point when the study was conducted was 3.35 per 100 (95% CI: 3.16, 3.54). Similarly, for

the ulcers with diagnostic confirmation, the prevalence was 2.56 per 100 (95% CI: 2.39-2.73) (table 1). Alternatively, the prevalence of peptic ulcers at any point in the past was 10.28 per 100 (95% CI: 9.94, 10.63). Also, for peptic ulcers with diagnostic confirmation at any point in the past, the prevalence was 7.71 per 100 (955 CI: 7.43, 8.00). The study also mentioned that duodenal ulcers occur 3 to 4 times more frequently than gastric ulcers, as reported by previous studies. Nonetheless, western societies have displayed a decline in prevalence over the past decades[17].

Table 1: U.S. prevalence of peptic ulcer, by ulcer history, type, and diagnostic confirmation: The 1989 National Health Interview Survey[17].

Ulcer History	Ulcer Type	All Physician-Diagnosed Ulcers			Ulcer with Diagnostic Confirmation			Subjects with Diagnostic Confirmation, %
		No. Subjects (millions)	Prevalence per 100	95% CI	No. Subjects (millions)	Prevalence per 100	95% CI	
Any time in the past	Gastric ulcer	4.29	2.44	2.26, 2.63	3.15	1.79	1.64, 1.94	73
	Duodenal ulcer	4.44	2.53	2.36, 2.71	4.07	2.31	2.15, 2.48	91
	Peptic ulcer of any type	10.04	5.64	5.38, 5.90	6.89	3.88	3.68, 4.10	69
	All types	18.30	10.28	9.94, 10.63	13.66	7.71	7.43, 8.00	75
Within the last year	Gastric ulcer	1.64	0.92	0.83, 1.02	1.25	0.70	0.61, 0.79	76
	Duodenal ulcer	1.26	0.71	0.62, 0.79	1.19	0.67	0.59, 0.75	94
	Peptic ulcer of any type	3.28	1.85	1.71, 1.97	2.34	1.31	1.19, 1.43	71
	All types	5.98	3.35	3.16, 3.54	4.57	2.56	2.39, 2.73	76

Note. The ulcer categories used in the present study were as follows: physician-diagnosed ulcers of all types, gastric ulcer with diagnostic confirmation, and duodenal ulcer with diagnostic confirmation. (Diagnoses were confirmed with an upper gastrointestinal series of x-rays or an upper endoscopy.) See the "Results" for sample sizes. CI = confidence interval.

The study also reported associations between peptic ulcers and other confounders. Peptic ulcers showed an age related rise in prevalence, especially between ages 65 to 74. Non Hispanic whites reported ulcers more frequently than other ethnicities. Also, individuals with a

8

divorced or separated marital status were associated with an increased prevalence of peptic ulcers. The same was true for veterans. There was an inverse relationship between income and prevalence, and education and prevalence. Smoking was strongly associated with all types of ulcers. Both ulcer types were shown to affect men more than women. More specifically, gastric ulcers for the previous 12 months were associated with lower education, lower income, smoking, and a divorced or separated status. Duodenal ulcers, on the other hand, were strongly associated with age and smoking[17].

Kang and colleagues reported an age-standardized period prevalence in England and Wales of peptic ulceration. In this study, peptic ulcers decreased between 1994 and 1998. For men, the prevalence decreased from 3.3 per 1000 to 1.5 per 1000, and for women the prevalence decreased from 1.8 per 1000 to 0.9 per 1000. Younger people showed a greater decline (Tables 2 and 3). The authors suggested that the observed decline, mostly seen in younger people, might be due to a widespread *H. pylori* eradication therapy. A slower decline observed in elders could be due to drug use to treat this condition. Peptic ulcers appear to be more commonly diagnosed in the USA than the UK, but a downward trend in prevalence is evident in both countries as well[18].

Table 2: Period prevalence of peptic ulceration among men in 1994 and 1998 showing a peptic ulcer prevalence decrease in England and Wales[18].

	0–34	35–44	45–54	55–64	65–74	75–84	85+	Age-standardized rate (all ages)
1994								
No. with peptic ulceration	271	273	371	370	257	105	22	1669
Period prevalence/1000 patients	1.1	3.8	5.5	7.4	6.3	5.4	4.8	3.3
Percentage prescribed H_2 antagonists	69.7	74.4	71.7	68.1	78.2	73.3	72.7	72.3
Percentage prescribed proton pump inhibitors	47.2	53.1	52.6	57.3	54.9	41.0	45.5	52.1
Percentage prescribed antacids	20.3	17.2	18.3	21.4	28.0	34.3	18.2	22.3
Percentage prescribed chelates	11.4	13.9	13.7	9.5	8.9	1.9	0.0	10.3
1998								
No. with peptic ulceration	121	164	169	184	169	134	29	970
Period prevalence/1000 patients	0.4	1.8	2.0	2.9	3.6	5.2	4.8	1.5
Percentage prescribed H_2 antagonists	46.3	44.5	39.1	41.3	45.0	33.6	31.0	41.2
Percentage prescribed proton pump inhibitors	64.5	73.8	76.9	75.5	71.6	76.1	79.3	73.3
Percentage prescribed antacids	14	18.3	11.2	19.6	26	29.1	20.7	18.8
Percentage prescribed chelates	0.8	3.0	1.2	3.3	1.2	3.0	0.0	2.0

Table 3: Period prevalence of peptic ulceration among women in 1994 and 1998 showing a peptic ulcer prevalence decrease in England and Wales[18].

	0–34	35–44	45–54	55–64	65–74	75–84	85+	Age-standardized rate (all ages)
1994								
No. with peptic ulceration	121	144	186	198	177	136	43	1005
Period prevalence/1000 patients	0.5	2.0	2.9	4.0	3.7	4.3	3.4	1.8
Percentage prescribed H_2 antagonists	67.8	70.1	68.8	77.8	71.8	64.7	60.5	70.8
Percentage prescribed proton pump inhibitors	43.8	53.5	52.7	54.0	51.4	57.4	41.9	51.6
Percentage prescribed antacids	28.9	29.2	19.9	31.3	35.0	44.9	37.2	30.4
Percentage prescribed chelates	5.8	11.1	14.5	9.1	7.9	4.4	2.3	9.3
1998								
No. with peptic ulceration	69	78	117	113	136	107	40	660
Period prevalence/1000 patients	0.3	0.9	1.4	1.8	2.5	2.7	2.5	0.9
Percentage prescribed H_2 antagonists	49.3	51.3	38.5	53.1	35.3	40.2	37.5	44.7
Percentage prescribed proton pump inhibitors	58.0	73.1	75.2	76.1	77.2	76.6	65.0	73.2
Percentage prescribed antacids	20.3	24.4	22.2	33.6	30.1	37.4	25.0	27.6
Percentage prescribed chelates	2.9	1.3	0.0	2.7	0.0	2.8	2.5	1.8

Peptic ulcer perforation can also occur in 2% to 10% of peptic ulcers[19]. Perforation

involves 60% of the time the anterior wall of the duodenum, 20% of the time it might occur due

to antral gastric ulcers, and 20% of the time due to lesser curve gastric ulcers. Such condition

can lead to a life threatening peritoneal perforation that consequently causes a chemical and

bacterial peritonitis[1]. Older patients can have up to 30% to 50% mortality rates due to

perforation and its management[20].

Peptic ulcer diagnosis

Several tests exist to identify gastric ulcers. The esophagogastroduodenoscopy (EGD) is

recommended for patients that have bleeding, weight loss, persistent vomiting, unresponsive

symptoms to medications, and people older than 55 years. EGD has a 90% sensitivity and

specificity for gastric and duodenal ulcers. Barium or diatrizoate meglumine and diatrizoate

sodium contrasts radiography is another diagnostic method for peptic ulcers and indicated

when an endoscopy is not possible or in the event of gastric outlet obstructions. This technique

has 80% to 90% sensitivity in detecting duodenal ulcers[1].

***Helicobacter pylori* ulcers**

Despite the fact that most patients with a peptic ulcer condition also contain *H. pylori*,

only about 10-15% of those inflicted with *H. pylori* develop such gastrointestinal condition[21]. It

is speculated that the bacteria bind to the gastric mucosa. Some environmental factors such as

developmental immaturity, infection, and malnutrition affect the parietal cell mass and acid secreting capacity. As a result, they become predisposing factors for gastric ulcers in response to *H. pylori*[22]. Together with the presence of an outer inflammatory protein and a cytotoxin-associated gene in the bacterial chromosome, the virulence is increased and therefore the ulcerogenic potential increases[23]. An infection with *H. pylori* increases the gastrin levels and reduces the gastric mucus production together with the duodenal mucosal bicarbonate secretion. One hypothesis suggests that *H. pylori* produce a high concentration of ammonia through urease activity and consequently prevents the antral D cells from sensing acidity in the antrum. This disrupts the inhibitory control of gastrin release. Alternatively, another hypothesis suggests that this bacterium may affect the D cells and G cells through stimulated production of cytokines[24]. Therefore, an eradication of this bacterium significantly reduces the recurrence of peptic ulcer from 67% to 6% in duodenal ulcer patients, and 59% to 4% in gastric ulcer patients[25].

Multiple tests exist to detect *H. pylori*. Serologic ELISA is a useful technique for initial testing, but cannot be used to confirm eradication. It has an 85% sensitivity, and a 79% specificity. The urea breath test is also used and can confirm eradication, but it is more expensive; however, it has a 95% to 100% sensitivity, and 91% to 98% specificity. The stool antigen test is also used and can confirm eradication, although it might be inconvenient for the patient. It has a 91% to 98% sensitivity, and a 94% to 99% specificity. The urine based ELISA and rapid urine test is another method used to diagnose, but it cannot confirm eradication; however, it has a 70% to 96% sensitivity, and a 77% to 85% specificity. The last method

available for diagnosis of this bacterium is the endoscopic biopsy. When it is performed with a

culture, it has a 70% to 80% sensitivity, and a 100% specificity; histologically, it has more than a

95% sensitivity, and 100% specificity; with a rapid urease test it has a 93% to 97% sensitivity,

and a 100% specificity[1].

H. pylori mode of infection and age when host is infected

Four models of *H. pylori* transmission have been determined largely through

seroprevalence studies[26]. Mostly, researchers concur that infection can occur through person to

person transmission[27,28]. However, there is also evidence supporting the fecal to oral and oral

to oral routes of infection[29-34]. Additional studies have found support for the waterborn

transmission route of infection[34-38].

Colonization of *H. pylori* happens at an early age. In animal study of ferrets, colonization

of the related parasite *H. mustelae* was determined at a young age. This happens usually

between the 5th and 6th weeks of age[39]. Similarly, studies in humans of developing countries

and lower socioeconomic strata have reported early colonization with *H. pylori*[40,41]. Findings in

the study of ferrets suggest that *H. mustelae* as well as *H. pylori* can induce glandular necrosis

or atrophy because the mode of infection in ferrets by their related parasite is similar to the

one in humans. Histologic findings in ferrets show that H. mustelae, as in the case of H. pylori,

colonizes faveolar epithelium by adhering tightly to gastric epithelial cells[39].

NSAIDs ulcers

Without the presence of *H. pylori*, NSAIDs are the most common cause of peptic

ulcers[42]. They cause a submucosal erosion, and inhibit formation of prostaglandins and their

protection from cyclooxygenase 2-mediated effects. These effects stimulate bicarbonate

secretion to protect the gastric mucosa, and stimulate epithelial cell proliferation to increase

the blood flow of the mucosa[43]. Patients who use NSAIDs long term have a 1% to 4% risk of

developing a life threatening ulcer complication, and the use of NSAIDs is also responsible for

one half of perforated ulcers. These complicated ulcers through NSAIDs occur more often in

elder patients[44,45,46].

NSAID mechanism of action

NSAIDs are drugs that contain analgesic, antipyretic, and in higher doses anti-

inflammatory effects. Examples of such drugs include aspirin, ibuprofen, and naproxen. They

inhibit the enzyme cyclooxygenase (COX) non selectively, and therefore have an effect on the

isoenzymes cyclooxygenase 1 (COX 1) and cyclooxygenase 2 (COX 2). COX 1 has clear

physiological functions, such as production of prostacyclin that has an antithrombogenic effect

when released by the endothelium and it is cytoprotective when released from the gastric

mucosa. On the other hand, COX 2 is induced in cells by stimuli that favor inflammation. It is

suggested that NSAIDs anti-inflammatory actions are attributed to COX 2 inhibition, while the

undesired side effect of stomach irritation is due to COX 1 inhibition (figure 3)[47]. Arachidonic

acid that originates from the phospholipid bilayer of cells through the catalytic effect of

phospholipase A_2 is metabolized by COX into prostaglandins and thromboxane (Figure 4).

Prostaglandin is produced in response to trauma and has a myriad of function such as

constriction or dilation in vascular smooth muscle, aggregation or disaggregation of platelets,

and sensitizing neurons to pain among others. Thromboxane is a vasoconstrictor, potent

hypertensive agent, and facilitates platelet aggregation[48].

Figure 3: Site of NSAID metabolic action[47].
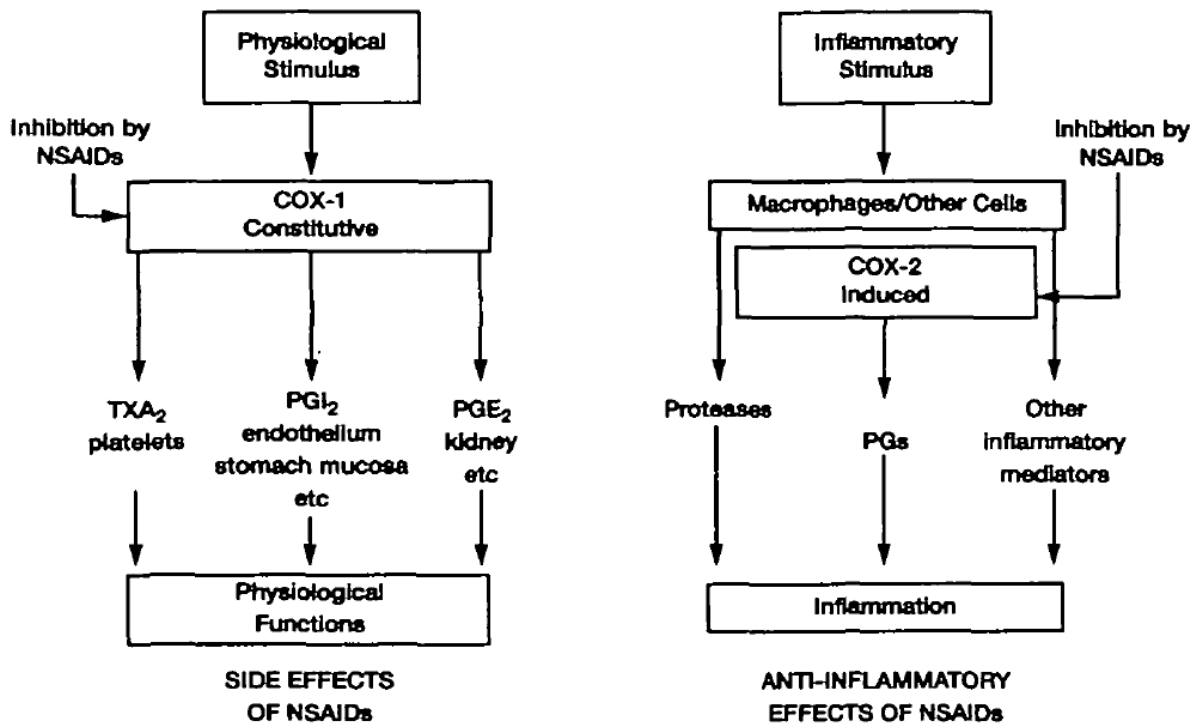
Figure 4: Site of action of aspirin in the catabolic pathway of arachidonic acid. AA: Arachidonic acid; PG: Prostaglandin; TBX: Thromboxane; LT: Leukotrines; 5-HPETE: 5-hydroperoxyeicosatetraenoic acid (arachidonic acid 5-hydroperoxide; HETE: Hydroxyeicosatetraenoic acid[47].
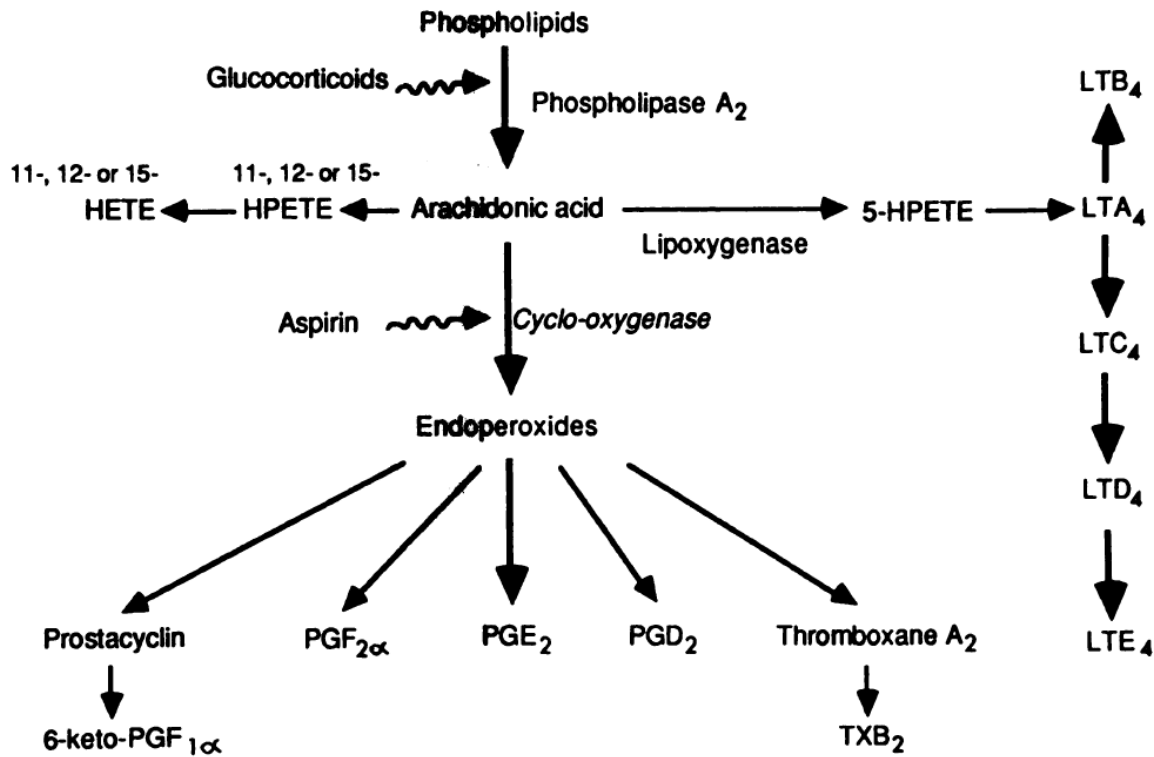
Non *H. pylori* and non NSAIDs ulcers

The prevalence of *H. pylori* infections has been in the decline in developing countries. One study conducted in the U.S. reported that 47% of white patients and 22% of non-white patients with ulcers were not using NSAIDs and tested negative for an *H. pylori*[2]. Since ulcers related to this bacterium are declining, ulcers related to other causes will likely remain stable

16

because they are unaffected by declines in levels of *H. pylori* . Therefore, in the future a greater proportion of *non-H. pylori* and non-NSAIDs ulcers will be diagnosed, and that will have meaningful implications in clinical management. The *H. pylori* test-and-treat commonly used strategy for dyspepsia, for instance, might not be cost efficient any longer, since the positive predictive value will fall for *H. pylori* testing. Moreover, a larger quantity of patients might need maintenance acid suppressive therapy to prevent ulcer recurrence[49].

Treatment of peptic ulcer

Several methods are used to treat peptic ulcer condition. First, *H. pylori* need to be eradicated, if present. Several, combinations of antibiotics with other medications are used to treat this bacterium, which have eradication rates of 80% to 90% or higher. The treatment usually lasts for 10 to 14 days. Histamine H_2 blocker is successful in healing 70% to 80% of duodenal ulcers after a four week treatment regimen, and an 87% to 94% after an eight week regimen. Proton pump inhibitors have an 80% to 100% healing rate after a treatment regimen of four weeks in the case of duodenal ulcers, and eight weeks for gastric ulcers. Sucrate (Carafate) has an effective healing rate like the H_2 blockers with a treatment regimen of four weeks. Lastly, surgery is used, but it is rarely needed. In the case of duodenal ulcers, truncal vagotomy, selective vagotomy, highly selective vagotomy, and partial gastrectomy are performed. Similarly, in the event of gastric ulcers, partial gastrectomy with gastroduodenal or gastrojejunal anastomosis is performed[50,51-55]. In cases of perforated gastric ulcers, treatment is

conducted through a meal patch, wedge resection of the ulcer, or a partial gastrectomy and reanastomosis[1].

METHODS

The data for this cross sectional study are from the National Health and Nutrition Examination Survey (NHANES) 1999-2000 online public use databases. NHANES is a program of studies intended to assess the health and nutritional status in the U.S. of adults and children. This database contains interviews and physical examinations and is a major part of the National Center for Health Statistics (NCHS), which is part of the Centers for Disease Control and Prevention (CDC). It is responsible for generating vital and health statistics for the United States[56]. Starting in 1999, NHANES became a continuous survey every two years, rather than the periodic one. Since 2003, the contents for each two year period are held mostly constant[57]. We are conducting the first study evaluating a possible association between RBC folic acid levels and history of peptic ulcers among people 20 to 80 years of age who participated in NHANES during the years 1999-2000.

Data Collection

NHANES personnel collect data in various ways. First a health care interviewer contacts a randomly selected home, where the interviewer determines eligibility. If the household is

eligible, the interviewer asks questions regarding health history. An appointment is then scheduled by the interviewer for the household to participate in a one-time health exam at a specially designed and equipped mobile center. Mobile centers travel to various locations throughout the country. The mobile center personnel consist of a physician, health and medical technicians, and dietary and health interviewers. Many speak English and Spanish to facilitate data collection. State of the art tools are utilized by these personnel and respondents to collect and process all the NHANES data that mostly avoids the need of paper forms and manual coding procedures. Interviewers use notebook computers with electronic pens, and mobile center staff transfer data automatically into databases. Respondents enter their answers to sensitive questions in complete privacy through a touch-sensitive computer screen. The collected survey information is readily available within 24 hours to NCHS staff. These methods enhance the quality of the data and increase the speed of data release[56].

Data Quality Assurance and Control

Quality assurance and control of the questionnaire data are provided through the NHANES computer-assisted personal interview (CAPI) software program, and through review from the NHANES field office staff. This software is used to collect data and has a built-in data edit and consistency check. When unusual or potentially erroneous data are recorded, the data edit check alerts the interviewer. The consistency check alerts the interviewer as well when observed inconsistencies arise, which the software compares with previous data entries or characteristics of the respondent. Skip patterns are also programmed in CAPI to reduce the

burden on the respondent. Notebook computers provide terminology descriptions and concepts used in the questionnaires for the interviewer, if needed. Once the data are collected, records of the interview are reviewed by the NHANES field office staff to determine accuracy and completeness. They also re-contact a subset of those interviewed to verify the information. The NCHS also participates together with contractor staff in the data validation process by evaluating recorded interviews[57].

Other methods that have been used by NHANES to validate data involve lab procedures or standardized questionnaires to assess mental disorders and stress. RBC folic acid and *H. pylori* measurements were obtained and their measures validated through lab procedures. Measures of stress in the form of panic disorders, major depression, and generalized anxiety were collected by means of the World Health Organization Composite International Diagnostic Interview, Version 2.1 (CIDI-Auto 2.1). CIDI is an entirely standardized and comprehensive interview utilized to evaluate mental disorders and provide diagnoses (Apendix I and II).

NHANES notifies local government officials in advance in each of the locations where a coming survey will take place. All households in the study area of interest receive a survey introductory letter from the NCHS director. The local media help in advertising the survey. The survey procedure is designed to ease and encourage participation by providing transportation to the mobile centers, if necessary. Respondents also receive compensation and a final data analysis report after their participation. Prior to their participation, subjects sign an informed consent, and all the information collected is entirely confidential to comply with the law[56]. Only

a sequence number is assigned to each participant, which is used to merge various data files together, and to uniquely and confidentially identify each participant from the survey[58].

The data collected are then made available through publications in the scientific literature, the internet, and CD ROMs containing the data. People who benefit from these data are mostly research organizations, universities, health care providers, educators, as well as federal agencies. The main federal agencies who benefit are the Food and Drug Administration (FDA), the National Institute of Health (NIH), CDC, and the United States Department of Agriculture (USDA). The FDA collaborates in the design and development of the survey. The NIH, FDA, and CDC use NHANES data for implementation and evaluation of their programs. The USDA together with NCHS report dietary and nutrition information from the data collected in these surveys. The United States Environmental Protection Agency (USEPA) also partners with NHANES to evaluate environmental influences on health[56].

Data Analysis

NHANES data are made available in Statistical Analysis System (SAS) format. The data were imported into STATA 11. Subjects were classified as cases (1) if they reported having a peptic ulcer during their lifetime, and they were classified as controls (0) if they did not report having a peptic ulcer during their lifetime.

We considered all the data for people younger than 20 years of age as an exclusion criterion. This exclusion was necessary because most of the variables in the survey had

questions that excluded people younger than that age. To be consistent, we excluded the data of the variables that included people younger than 20 years. Also, we excluded the data of people who responded to any of the questionnaire questions as refused or do not know.

Study Population

Data were collected by NHANES on 9965 individuals. The total sample population size provided by NHANES 1999-2000 accounts for all ages. We used an analytic subset of those who had data for both lifetime peptic ulcers and RBC folic acid, which was 2,433 for ages 20 and above (Figure 5)

NHANES over-samples persons 60 years and older, African Americans and Hispanics to produce adequate statistics[57]. NHANES utilizes various forms of survey weights. Data are weighted accordingly to generalize it to people. Four year weights and two year weights are available. We used a two year sample weight for this analysis. A stratum variable and a primary sample unit variable also provided in the NHANES 1999-2000 database were used. The primary sampling units are single counties or a combination of small counties, as long as they meet a minimum population size [59](Apendix III). PSUs are combined to form strata. A series of neighborhoods represents a stratum [60].

Figure 5: Study sample size obtained from NHANES 1999-2000

Number of people participating in NHANES 1999-2000
9965 (100%)[*]

Number of people 20 years or older with a blood sample test for RBC folic acid from NHANES 1999-2000. (Main exposure variable)
4880 (49%)[*]

Number of people 20 years or older with lifetime peptic ulcers from NHANES 1999-2000. (Main outcome variable)
2433 (24.4%)[*]
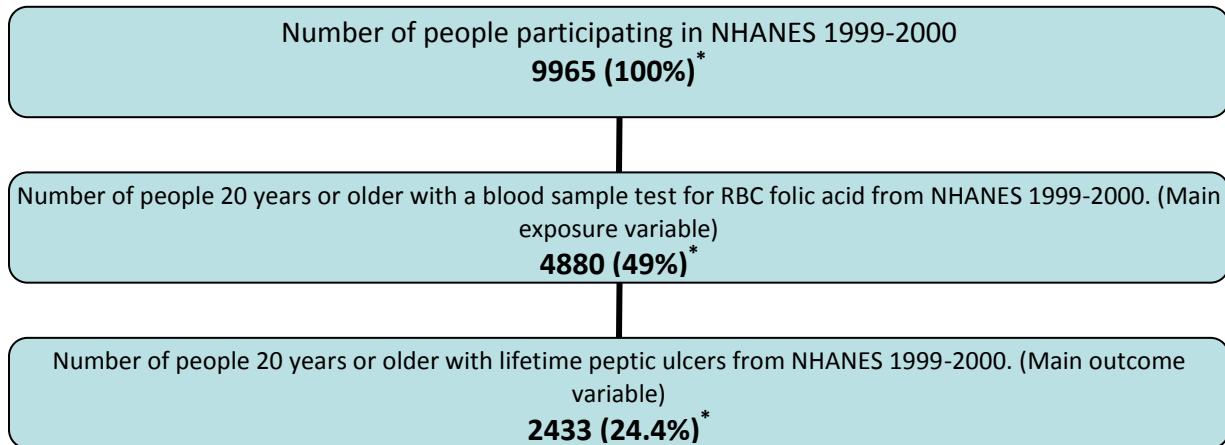
*Number of respondents and unweighted response rate per total number of participants, main exposure variables, and main outcome variable.

Study Variables

RBC folic acid, the main exposure variable, was provided as continuous in the NHANES 1999-2000 dataset. We categorized it in quartiles based on literature recommendations and number of respondents per strata[4]. We tried to keep similar numbers for each stratum for analysis purposes. Those with low numbers were merged with another stratum.

For variables that had missing data to account for all participants who had a lifetime peptic ulcer, an additional "missing" stratum was created to account for them. We did not use the "missing" stratum in variables that did not have sufficient data for this stratum in further statistical calculations. The variables that did not include the missing stratum in later statistical calculations were RBC folic acid, gender, ethnicity, age in years, veteran/military status and

level of education (table 4 and 7). The dependent variable was in the YES/NO dichotomous form, and therefore we were able to use a logistic regression in the analysis.

Main Outcome Variable

Regarding self reported lifetime peptic ulcers the information was collected by CDC staff members through household interviews. We coded participants who answered yes to the question "have you ever been told by a doctor or health care professional that you/s/she had an ulcer? This could be stomach, duodenal or peptic ulcer," as having peptic ulcers (1). By contrast, we coded those who answered no as not having peptic ulcers (0). We coded others who refused to answer or did not know, as not available (Table 4).

Main Explanatory Variable

CDC staff members collected information regarding RBC folic acid in specially designed and equipped mobile centers. Blood samples were collected to determine RBC folate levels, and results ranged from 28 ng/ml to 2352 ng/ml. We coded those who had levels at or below 227 ng/ml as 1, between 228 ng/ml and 301 ng/ml as 2, between 302 ng/ml and 628 ng/ml as 3, and above or at 620 ng/ml as 4. We coded those who did not have an RBC folate blood sample, but did respond to the main outcome variable question as 999 and missing. By contrast, we coded those who did not answer to the main outcome variable question, nor the main predictor variable question, as not available (Table 4).

24

Other Variables

  H.pylori was as a confounding variable that we selected. CDC staff members collected information through blood samples in specially equipped and designed mobile centers. These blood samples were analyzed through an enzyme linked immuno-sorbent assay (ELISA), and an immune status ratio (ISR) determined. This ratio ranged from 0 to 5.73. We coded those who had levels at or below 0.2 as 1, between 0.2 and 0.38 as 2, between 0.39 and 1.72 as 3, and above 1.72 as 4. We coded those who answered to the main outcome variable question, but did not have an *H.pylori* ELISA blood sample test as 999 and missing. Also, we coded those who did not answer to the main outcome variable question and neither had an *H.pylori* ELISA blood sample test as not available (table 4).

  NSAIDs products taken was another confounding variable that we selected. CDC staff members collected information on this variable through household interviews. We coded ibuprofen, feldene, and voltarin as 1. Also, we coded aspirin, Tylenol, and Excedrin as 2. We coded those who answered to the main outcome variable question, but did not answer to the question "which products have you taken?" as 999 and missing. Meanwhile we coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

  Gender was another confounding variable that we considered. CDC staff members collected information on this variable through household interviews. We coded male as 1, and female as 2. We coded participants who answered to the main outcome variable question, but did not answer to the question "gender of sample person?" as 999 and missing. Alternatively,

we coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

Marital status was another confounding variable of our consideration. CDC staff members collected information on this variable through household interviews. We coded married as 1, widowed as 2, divorced as 3, separated as 4, never married as 5, and living with partner as 6. We coded those who answered to the main outcome variable question, but did not answer to the question "marital status of sampled person?" as 999 or missing. We also coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table4).

Ethnicity was also a confounding variable that we considered. CDC staff members collected information on this variable through household interviews. We coded Mexican American as 1, Other Hispanic as 2, Non-Hispanic White as 3, Non-Hispanic Black as 4, and Other Race including Multi Racial as 5. We coded those who answered to the main outcome variable question, but did not answer to the question "race and ethnicity?" as 999 or missing. We also coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

Age was another confounding variable that we considered. CDC staff members collected information on this variable through household interviews. Age ranged from 0 years to 85 years; however, participants younger than 20 years were omitted in this study. We coded ages between 20 years and 34 years as 1, 35 years and 49 years as 2, 50 years and 67 years as 3, and anything above 68 years as 4. We coded those who answered to the main outcome variable

question, but did not answer to the question "age in years of the sample person at time of screening? Individuals 85 and over are top coded at 85 years of age," as 999 and missing. We also coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

Level of education was another confounding variable of our consideration. CDC staff members collected information on this variable through household interviews. We coded education less than 9th grade as 1, between 9th, 11th, and 12th grade with no diploma as 2, higher school grad/GED or equivalent as 3, some college or AA degree as 4, and college graduate or above as 5. Those who responded to the main outcome variable question, but did not respond to the question "what is the highest grade or level of school completed or the highest degree you have s/he has received?" as 999 and missing. We also coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

Smoking (someone smokes at home) as a confounding variable of our consideration. CDC staff members collected information on this variable through household interviews. We coded participants who answered yes as 1, and no as 0. We coded those who answered to the main outcome variable question, but did not answer to the question "does anyone smoke in the home?" as 999 and missing. We also coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

Alcohol consumption (number of alcoholic drinks in the past month) was another confounding variable that we considered. CDC staff members collected information on this variable through household interviews. The data ranged from 1 drink to 36 drinks per day. We coded one or less drinks per day as 1, 2 drinks per day as 2, and 3 to 40 drinks per day as 3. We coded those who answered to the main outcome variable question, bud did not answer to the question "in the past 12 months, on those days that you drank alcoholic beverages, on the average, how many drinks did you/he/she drink?" as 999 and missing. We also coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

Stomach or intestinal illness (with vomiting and diarrhea in the past 30 days) was another confounding variable that we considered. CDC staff members collected information on this variable through household interviews. We coded participants who answered yes as 1 and no as 0. We coded those who answered to the main outcome variable question, but did not answer to the question "did you have a stomach or intestinal illness with vomiting or diarrhea that started during those 30 days?" as 999 and missing. We also coded those who did not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

Income was another confounding variable worthy of our consideration. CDC staff members collected information on this variable through household interviews. We coded participants who answered and reported an income between $0 and $4,999 as 1, between $10,000 and $14,999 as 2, between $15,000 and $19,000 as 3, between $20,000 and $24,999

as 4, between $25,000 and $34,999 as 5, between $35,000 and $44,999 as 6, between $45,000

and $54,999 as 7, between $55,000 and $74,000 as 8, and above $75,000 as 9. We coded those

who answered to the main outcome variable question, but did not answer to the question

"total household income (annual)?" as 999 and missing. We also coded those who did not

answer to the main outcome variable question, nor the question pertaining to this confounding

variable, as not available (table 4).

Veteran/military status was another confounding variable that we considered. CDC staff

members collected information on this variable through household interviews. We coded

participants who answered yes as 1, and those who answered no as 0. We coded those who

answered to the main outcome variable question, but did not answer to the question "did you

ever serve in the Armed Forces of the United States?" as 999 and missing. We also coded those

who did not answer to the main outcome variable question, nor the question pertaining to this

confounding variable, as not available (table 4).

People per household was the last confounding variable that we considered for this

study. CDC staff members collected information on this variable through household interviews.

Responses ranged from 1 to 6. We coded households with one inhabitant as 1, households with

2 inhabitants as 2, households with 3 inhabitants as 3, households with 4 inhabitants as 4,

households with 5 inhabitants as 5, and households with 6 inhabitants as 6. We coded those

who answered to the main outcome variable questions, but did not answer to the question

"total number of people in the household?" as 999 and missing. We also coded those who did

not answer to the main outcome variable question, nor the question pertaining to this confounding variable, as not available (table 4).

Statistical Methods

To account for sampling weights we used STATA 11, and followed the analytic guidelines recommended by NHANES (Appendix III). To determine the appropriate settings for the data provided in NHANES 1999-2000, we used the sample weight variable titled "wtmec2yr". This variable accounts for the 2 year sampling period between 1999 and 2000 used in this study. To account for the strata we used the variable titled "sdmvstra," and to account for the primary sampling units we used the variable titled "sdmvpsu."

We identified several interactions from literature sources. We assessed *H. pylori* and NSAID consumption using statistical methods for their significance as effect modifiers. The statistical significance of each interaction was a determining factor for its addition into the final statistical model (Table 8).

We developed a statistical model after assembling a subset of variables acquired from NHANES 1999-2000 database through various statistical methods of variable selection (Tables 7,9,10,11). To assess the hypothesis, the dependent variable was history of peptic ulcer within a lifetime, while the independent variable was RBC folic acid. We added a number of confounder variables to the model, mainly H. pylori and NSAID consumption. We assessed the latter two as potential effect modifiers (table 8). We tested other confounders of interest for potential

inclusion in the model, such as gender, income, marital status, ethnicity, age, level of education, smoking, alcohol drinking, stomach or intestinal illness, military status, people per household, and NSAIDs (Table 4).

An additional manual and weighted statistical forward assessment analysis was conducted in the variable selection process. This analysis describes the changes that occur during this selection process beginning with the primary predictor variable, RBC folic acid. This analysis ended after including all the potential variables that would be included in a final statistical model, and by reporting on odds ratio and p-values changes for each variable at each step of variable addition (table 11).

Table 4: List of variables selected based on literature sources and available in NHANES 1999-2000

NHANES Measure	Measure Method	Possible Responses	Coding for Analysis
Lifetime Peptic Ulcers	Questionnaire: Have you ever been told by a doctor or health care professional that you/s/he had an ulcer? this could be stomach, duodenal or peptic ulcer.	1) Yes 2) No 7) Refused 9) Don't know (.) Missing	1) outcome 2) no outcome (.) Not available
RBC folic acid (ng/ml)	Blood sample: Folate, RBC (ng/ml RBC).	Range: 28 ng/ml to 2352 ng/ml (.) Missing	1) ≤ 227 ng/ml 2) ≥228 & ≤301 ng/ml 3) ≥302 & ≤628 ng/ml 4) ≥620 ng/ml 999) Missing (.) Not available

NHANES Measure	Measure Method	Possible Responses	Coding for Analysis
H. pylori (ELISA Immune Status Ratio (ISR))	Immunologic assay (ELISA)[*]: Helicobacter pylori.	Range: 0 to 5.73 (.) Missing	1) ≤ 0.2 2) >0.2 & ≤0.38 3) >0.38 & ≤1.72 4) >1.72 999) Missing (.) Not avaliable
NSAIDs (Products taken)	Questionnaire: Which products have you taken?	10) Aspirin 11) Tylenol 12) Ibuprofen 13) Excedrin 14) Vanquish 15) Feldene 16) Voltarin 17) Clinoril 18) Indocin 19) Naprosyn 20) Tolectin 77) Refused 99) Don't know (.) Missed	1) Ibuprofen, Feldene, Voltarin 2) Aspirin, Tylenol, Excedrin. 999) Missing (.) Not avaliable
Gender	Questionnaire: Gender of sampled person.	1) Male 2) Female (.) Missing	1) Male 2) Female 999) Missing (.) Not avaliable
Marital Status	Questionnaire: Marital status of sampled person.	1) Married 2) Widowed 3) Divorced 4) Separated 5) Never married 6) Living with partner 77) Refused 99) Don't know (.) Missing	1) Married 2) Widowed 3) Divorced 4) Separated 5) Never married 6) Living with partner 999) Missing (.) Not available
Ethnicity	Questionnaire: Race and ethnicity.	1) Mexican American 2) Other Hispanic 3) Non-Hispanic White 4) Non-Hispanic Black 5) Other Race-Including Multi Racial (.) Missing	1) Mexican American 2) Other Hispanic 3) Non-Hispanic White 4) Non-Hispanic Black 5) Other 999) Missing (.) Not available

NHANES Measure	Measure Method	Possible Responses	Coding for Analysis
Age (years)	Questionnaire: Best age in years of the sample person at time of screening. Individuals 85 and over are top coded at 85 years of age.	Range 0 to 85) 0 to 85 85) 85 (.) Missing	1) ≥20 & ≤34 2) ≥35 & ≤49 3) ≥50 & ≤67 4) ≥68 999) Missing (.) Not available
Level of Education	Questionnaire: What is the highest grade or level of school completed or the highest degree you have/s/he has received?	1) Less than 9th grade 2) 9-11th grade (includes 12th grade with no diploma) 3) High School grad/GED or equivalent 4) Some college or AA degree 5) College graduate or above 7) Refused 9) Don't know (.) Missing	1) ≤9th grade 2) 9-11th grade 3)High School Grad/GED or equivalent 4) Some College or AA degree 5) College Graduate or Above. 999) Missing (.) Not available
Smoking (Someone smokes at home)	Questionnaire: Does anyone smoke in the home?	1) Yes 2) No 7) Refused 9) Don't know (.) Missing	1) Yes 0) No 999) Missing (.) Not available
Alcohol Consumption (Number of alcoholic drinks in the past month)	Questionnaire: In the past 12 months, on those days that you drank alcoholic beverages, on the average, how many drinks did you/he/she have?	Range 1 to 36: 1 to 36 77) Refused 99) Don't know (.) Missing	1) ≤1 2) 2 3) ≥3 & ≤40. 999) Missing (.) Not available
Stomach or Intestinal Illness (with vomiting and diarrhea in the past 30 days)	Questionnaire: Did you have a stomach or intestinal illness with vomiting or diarrhea that started during those 30 days?	1)Yes 2) No 7) Refused 9) Don't know (.) Missing	1)Yes 0) No 999) Missing (.) Not available

NHANES Measure	Measure Method	Possible Responses	Coding for Analysis
Income	Questionnaire: Total household income (annual).	1) $0 to $ 4,999 2) $5,000 to $9,999 3) $10,000 to $14,999 4) $15,000 to $19,999 5) $20,000 to $24,999 6) $25,000 to $34,999 7) $35,000 to $44,999 8) $45,000 to $54,999 9) $55,000 to $64,999 10)$65,000 to $74,999 11)$75,000 and over 12) Over $20,000 13) Under $20,000 77) Refused 99) Don't know (.) Missing	1) $0-9,999 2) $10,000-14,999 3) $15,000-19,999 4) $20,000-24,999 5) $25,000-34,999 6) $35,000-44,999 7)$45,000-54,999 8)$55,000-74,999 9) ≥$75,000 999) Missing (.) Not available
Veteran/Military Status	Questionnaire: Did you ever serve in the Armed Forces of the United States?	1) Yes 2) No 7) Refused 9) Don't know (.) Missing	1)Yes 0)No 999) Missing (.) Not available
People per Household	Questionnaire: Total number of people in the household.	Range: 1 to 6) 1 to 6 7) 7 or more people in the household. (.) Missing	1) 1 2) 2 3) 3 4) 4 5) 5 6) 6 999) Missing (.) Not available

Consult appendix I for NHANES data collection techniques for the variables selected in this study.
*Consult appendix I on ELISA techniques for *H. pylori* measures.

RESULTS

We evaluated the data through several statistical analyses. First we reported baseline

characteristics of the outcome variable (table 5), response rates per variable (table 6), and

baseline characteristics of variables by output (table 7). Then we reported on an assessment of interactions using sample weights (table 8), a univariate logistic regression association between the main outcome variable and each confounding variable using sample weights (table 9), a confounding assessment using sample weights (table 10), a step by step summary of multiple logistic regression model using sample weights (table 11), and am multivariate logistic regression association between the outcome variable and the main predictor variable (Table 12).

Demographics:

Among a total of 9965 people who participated in the NHANES 1999-2000 survey. For the purpose of this analysis 2,433 people 20 to 80 years of age responded to whether he or she was ever told of having stomach, duodenal, or peptic ulcer in a lifetime. Among those 2,433, 256 responded to having a peptic ulcer in their lifetime. Meanwhile 2177 responded no to ever having a peptic ulcer in their lifetime (Table 5). Thus, the weighted prevalence of lifetime peptic ulcers in NHANES 1999-2000 was 9.9%. The unweighted response percent was 24.4% for the main predictor variable of lifetime peptic ulcers. An unweighted 49% response was seen for levels of folic acid, levels of *H. pylori*, gender, ethnicity, age, and people per household. Other variables, such as NSAIDs use had an unweighted 5.9% response rate, marital status an unweighted 43.5% response rate, level of education an unweghted 48.7% response rate, smoking an unweighted 48.1% response rate, alcohol consumption an unweighted 12.8% response rate, stomach or intestinal illness an unweighted 40.7% response rate, income an

unweighted 40.95% response rate, and veteran and military status an unweighted 48.85%

response rate (Table 6).

Study Variables

Each variable was then evaluated by outcome of history of peptic ulcers. The variables

containing the lowest numbers of sample units were NSAID with 309, and alcohol drinking with

682. The numbers within these two variables were demonstrated to be too small for a

meaningful statistical analysis. On the other hand, RBC folic acid, H. pylori, gender, ethnicity,

and age had a total number of respondents by outcome of 2,433. Marital status had a total

number of respondents by outcome of 2,163, level of education had a total of 2,425 by

outcome, smoking had a total of 2, 386 by outcome, stomach or intestinal illness had a total of

2,120 by outcome, income had a total of 2,043 by outcome, and veteran and military status had

a total of 2,429 by outcome. A category of missing sample units was established for each

variable by outcome in order to account for those who answer to the main outcome variable

question, but did not answer to a confounding variable question. In this manner, now all

variables display a total of 2433 sample units. Those who do not meet that total, show the

number of missing units needed to add up to that number (table 7).

Table 5: Outcome variable baseline characteristics, NHANES 1999-2000.

NHANES Measure	Total Respondents (N) (Unweighted)	Percent of Participants (Weighted)
Lifetime Peptic Ulcers	2,433	100%
Outcome	256	9.9%
No outcome	2177	90.1%
Refused	1 (excluded)	excluded
Don't know	12 (excluded)	excluded

Table 6: Unweighted response rates of 20 years of age and older for the variables selected from literature sources as potential fit for a final logistic regression analysis, NHANES 1999-2000.

NHANES Measure	Total Respondents	Response Rate[*]
Lifetime Peptic Ulcers	2,433	24.4%[¥]
RBC folic acid (ng/ml)	4,880	49%
H. pylori (ELISA Immune Status Ratio (ISR))	4,880	49%
NSAIDs (Products taken)	592	5.9%
Gender	4,880	49%
Marital Status	4,335	43.5%
Ethnicity	4,880	49%
Age (years)	4,880	49%
Level of Education	4,856	48.7%
Smoking (Someone smokes at home)	4,794	48.1%
Alcohol Consumption (Number of alcoholic drinks in the past month)	1,280	12.8%
Stomach or Intestinal Illness (with vomiting and diarrhea in the past 30 days)	4,062	40.7%

NHANES Measure	Total Respondents	Response Rate[*]
Income	4,081	40.95%
Veteran/Military Status	4,868	48.85%
People per Household	4,880	49%

*Refers to those who provided an answer to the question pertaining to each variable.

[¥]Pertains to the percentage of those who had or did not have a lifetime peptic ulcer.

Table 7: Baseline characteristics of variables by output, NHANES 1999-2000.

NHANES Measures	Total and Percent of Respondents by Outcome[*]			
	Total n(%)	Ulcer n(%)	No ulcer n(%)	p- value
RBC folic acid (ng/ml)	2,433(100%)	----	-----	0.9194
≤ 227	617(25.8%	73(2.7%)	544(23%)	
≥228 & ≤301	619(25.2%)	63(2.5%)	556(22.7%)	
≥302 & ≤628	656(26.8%)	60(2.4%)	596(24.4%)	
≥629	541(22.1%)	60(2.2%)	481(19.9%)	
Missing	-----	-----	-----	
***H. pylori* (ELISA Immune Status Ratio (ISR))**	2,433(100%)	-----	-----	0.0577
≤ 0.2	445(18.4%)	48(2.0%)	397(16.3%)	
>0.2 & ≤0.38	524(21.9%)	44(1.8%)	480(20.0%)	
>0.38 & ≤1.72	446(18%)	37(1.1%)	429(4.9%)	
>1.72	998(41.7%)	127(4.9%)	871(36.8%)	
Missing	-----	-----	-----	
NSAIDs (Products taken)	2,433	-----	-----	0.2362
Ibuprofen, Feldene, Voltarin	82(8.75%)	29(1.05%)	198(7.7%)	
Aspirin, Tylenol, Excedrin.	227(33%)	10(0.53%)	72(2.8%)	
Missing	2,124(87.9%)	217(8.4%)	1,907(79.6%)	
Gender	2,433(100%)	----	----	0.5047
Male	1,139(49.3%)	117(4.7%)	1,022(45.5%)	
Female	1,294(50.7%)	139(5.2%)	1,155(45.5%)	
Missing	-----	-----	-----	

NHANES Measures	Total and Percent of Respondents by Outcome[*]			
	Total n(%)	Ulcer n(%)	No ulcer n(%)	p- value
Marital status	2,433	-----	-----	0.9053
Married	1,204(50.3%)	123(4.7%)	1,081(45.5%)	
Widowed	224(4.4%)	18(0.4%)	206(4.0%)	
Divorced	178(7.5%)	16(0.7%)	162(6.8%)	
Separated	99(3.9%)	12(0.4%)	87(3.5%)	
Never married	352(16.8%)	35(1.7%)	317(15.1%)	
Living with partner	106(4.9%)	12(0.6%)	94(4.3%)	
Missing	270(12.28%)	40(1.45%)	230(10.8%)	
Ethnicity	2,433(100%)	----	----	0.2586
Mexican American	625(6.2%)	61(0.5%)	564(5.6%)	
Other Hispanic	163(8.3%)	20(1.1%)	143(7.1%)	
Non-Hispanic White	1,078(68.7%)	114(6.4%)	964(62.3%)	
Non-Hispanic Black	471(11.6%)	53(1.5%)	418(10.1%)	
Other	96(5.3%)	8(0.4%)	88(5%)	
Missing	-----	-----	-----	
Age (years)	2,433(100%)	-----	-----	0.5696
≥20 & ≤34	675(32.4%)	80(3.8%)	595(28.6%)	
≥35 & ≤49	588(32.4%)	53(3.8%)	535(28.6%)	
≥50 & ≤67	637(23.8%)	68(2.3%)	569(21.5%)	
≥68	533(11.4%)	55(1%)	478(10.4%)	
Missing	-----	-----	-----	
Level of education	2,433(100%)	-----	-----	0.7612
≤9th grade	473(7.4%)	46(0.6%)	427(6.8%)	
9-11th grade	497(17.4%)	57(2%)	440(15.4%)	
High School Grad/GED or equivalent	560(26.4%)	66(2.5%)	494(23.8%)	
Some College or AA degree	504(26.3%)	48(2.6%)	456(23.76%)	
College Graduate or Above	391(22.5%)	38(2.3%)	353(20.3%)	
Missing	8(0.01%)	1(0%)	7(0.01%)	
Smoking (Someone smokes at home)	2,433(100%)	-------	-------	0.3233
Yes	497(22.9%)	59(2%)	438(20.9%)	
No	1,889(75.6%)	192(7.9%)	1,697(87.8%)	
Missing	47(1.5%)	5(0.005%)	42(1.44%)	

NHANES Measures	Total and Percent of Respondents by Outcome[*]			
	Total n(%)	Ulcer n(%)	No ulcer n(%)	p- value
Alcohol drinking (Number of alcoholic drinks in the past month)	2,433(100%)	-----	-----	0.0906
≤1	224(9.3%)	30(1.53%)	194(7.7%)	
2	192(8.3%)	22(0.08%)	170(7.5%)	
≥3 & ≤40	266(11.27%)	28(1.1%)	238(10.2%)	
Missing	1,751(71.14%)	176(6.5%)	1,575(64.6%)	
Stomach or intestinal illness (with vomiting and diarrhea in the past 30 days)	2,433(100%)	-----	------	0.0343
Yes	199(8.5%)	30(1.4%)	169(7.1%)	
No	1,921(78.4%)	195(7.1%)	1,726(71.3%)	
Missing	313(13.1%)	31(1.4%)	282(11.7%)	
Income	2,433(100%)	----	-----	0.2892
$0-9,999	239(7.7%)	17(0.4%)	222(7.3%)	
$10,000-14,999	210(6.4%)	28(1.0%)	182(5.4%)	
$15,000-19,999	163(4.9%)	16(0.5%)	147(4.9%)	
$20,000-24,999	176(7.0%)	18(0.8%)	158(6.2%)	
$25,000-34,999	272(11.25%)	21(0.7%)	251(10.6%)	
$35,000-44,999	199(8.4%)	28(1.0%)	171(7.4%)	
$45,000-54,999	164(8.7%)	17(0.7%)	147(8.0%)	
$55,000-74,999	211(11.7%)	22(1.4%)	189(10.34%)	
≥$75,000	409(22.32%)	40(2.2%)	369(20.15%)	
Missing	390(11.6%)	49(1.4%)	341(10.2%)	
Veteran/Military Status	2,433(100%)	----	----	0.3205
Yes	344(14.8%)	44(1.8%)	300(12.9%)	
No	2,085(85.2%)	211(8.1%)	1,874(77.2%)	
Missing	4(0%)	1(0%)	3(0%)	

NHANES Measures	Total and Percent of Respondents by Outcome[*]			
	Total n(%)	Ulcer n(%)	No ulcer n(%)	p- value
People per Household	2,433(100%)	----	----	0.6388
1	363(13.1%)	42(1.8%)	321(11.3%)	
2	770(32.7%)	80(2.9%)	690(29.8%)	
3	452(20.9%)	45(1.9%)	407(19%)	
4	365(17.5%)	34(1.5%)	331(16%)	
5	231(9.0%)	27(1.1%)	204(7.9%)	
6	118(3.45)	13(0.4%)	105(3%)	
7	134(3.4%)	15(0.3%)	119(3%)	
Missing	----	----	----	

*Number of respondents by outcome (n) are unweighted, and percent (%) of participants by outcome are weighted.

Assessment of Interactions:

The potential for interactions was also tested to determine their need in a final statistical model. Two interactions were assessed, that of folic acid levels with gender, and folic acid levels with *H. pylori.* An interaction between NSAIDs use and folic acid levels was not pursued due to the low response rate in this variable. The interaction of folic acid with gender was not statistically significant (p=0.96; adjusted wald test), and the interaction of folic acid with *H. pylori* was also not statistically significant (p=0.09; adjusted wald test). Both interactions were therefore not considered for the final statistical model (Table 8).

Table 8: Interaction assessment using sample weights, NHANES 1999-2000.

Interaction	p-value (Adjusted Wald Test)
Folic acid and gender	0.96
Folic acid and *H. pylori*	0.09

Confounders:

We assessed thirteen variables as potential confounders in the association between lifetime peptic ulcers and RBC folic acid. For that purpose we evaluated variables that could confound the association. We tested these variables through a univariate statistical analysis, a confounder statistical assessment, and a manual statistical forward variable selection analysis (table 9, 10, and 11 respectively). The variables were RBC folic acid, *H.pylori* (ELISA Immune Status Ratio ISR), NSAIDs (products taken), gender, marital status, ethnicity, age (years), levels of education, smoking (someone smokes at home), alcohol drinking (number of alcoholic drinks in the past month), stomach or intestinal illness (with vomiting and diarrhea in the past 30 days), income, veteran/military status, and people per household. None of these potential confounding variables changed the odds ratios of RBC folic acid by 5% or more (table 10). Hence, none of these variables were considered to be confounders.

The univariate logistic regression analysis reported that all confounding variables were not statistically significant, except for income. RBC folic acid was not statistically significant (p=0.96), *H.pylori* (ELISA Immune Status Ration ISR) was not statistically significant (p=0.20), NSAIDs (products taken) was not statistically significant (p=0.20), gender was not statistically significant (p=0.50), marital status was not statistically significant (p=0.90), ethnicity was not

statistically significant (p=0.11), age was not statistically significant (0.36), level of education was not statistically significant (p=0.35), smoking (someone smokes at home) was not statistically significant (p=0.36), alcohol consumption (number of alcoholic drinks in the past month) was not statistically significant (p=0.21), stomach or intestinal illness (with vomiting and diarrhea in the past 30 days) was not statistically significant (p=0.10), income was the only statistically significant variable (p<0.00), veteran/military status was not statistically significant (p=0.32), and people per household was not statistically significant (p=0.80) (table 9).

The confounder statistical assessment did not show that the variables changed more than 5.01%. The crude odds ratios from the strata of the main predictor variable were compared with the adjusted odds ratio of the main predictor variable. The percent difference among the variables was between 0.02% and 5.01% (Table 10).

A step by step multiple logistic regression model using sample weights showed that the values for the main predictor variable did not change significantly after adding each of the 13 confounding variables considered for this study one by one. The main predictor variable was split in quartiles. The first quartile (≤ 227 ng/ml) was used as referent. The second quartile (≥228 & ≤301 ng/ml) showed a change in odds ratio from 0.93 (p=0.82) to 0.94 (p=0.83), the third quartile (≥302 & ≤628 ng/ml) showed a change in odds ratio from 0.84 (p=0.57) to 0.85 (p=0.62), and the fourth quartile (≥629 ng/ml) showed a change in odds ratio from 0.94 (p=0.78) to 0.89 (p=0.72) (table 11).

Two variables were excluded from the final logistic regression model. NSAID and alcohol consumption were discarded because of the very low response rate (table 7). These low

numbers posed significant difficulties in the statistical analysis process. The variables selected

for the final model were based on prior literature, especially those already used in a previous

study by Sonnenberg and others because there were no potential confounders that changed

the lifetime peptic ulcers and RBC folic acid association[17].

Table 9: Univariate logistic regression analysis: association between history of lifetime peptic ulcers using sample weights, NHANES 1999-2000.

NHANES Measure	Odds Ratio (95% CI) (weighted)	p-value (Pearson)
RBC Folic Acid (ng/ml)		
≤ 227	Referent	0.96
≥228 & ≤301	0.94 (0.51, 1.7)	
≥302 & ≤628	0.84 (0.43, 1.6)	
≥629	0.94 (0.59, 1.49)	
Missing	-------	
H. pylori (ELISA Immune Status Ratio (ISR))		
≤ 0.2	Referent	0.20
>0.2 & ≤0.38	0.75 (0.47, 1.19)	
>0.38 & ≤1.72	0.53 (0.30, 0.95)	
>1.72	1.09 (0.66, 1.77)	
Missing	------	
NSAIDs (products taken)		
Ibuprofen, Feldene, Voltarin	Referent	0.20
Aspirin, Tylenol, Excedrin.	1.40 (0.50, 3.90)	
Missing	0.77 (0.40, 1.48)	
Gender		
Male	Referent	0.50
Female	1.09 (0.82, 1.45)	
Missing	------	

NHANES Measure	Odds Ratio (95% CI) (weighted)	p-value (Pearson)
Marital status		
Married	Referent	0.90
Widowed	0.99 (0.49, 1.99)	
Divorced	0.98 (0.43, 2.24)	
Separated	0.99 (0.31, 3.15)	
Never married	1.06 (0.66, 1.70)	
Living with partner	1.36 (0.5, 3.69)	
Missing	1.28 (0.82, 1.99)	
Ethnicity		
Mexican American	Referent	0.11
Other Hispanic	1.71 (0.93, 3.15)	
Non-Hispanic White	1.09 (0.67, 1.79)	
Non-Hispanic Black	1.54 (0.98, 2.41)	
Other	0.81 (0.20, 3.19)	
Missing	------	
Age (years)		
≥20 & ≤34	Referent	0.36
≥35 & ≤49	0.74 (0.34, 1.60)	
≥50 & ≤67	0.83 (0.52, 1.33)	
≥68	0.71 (0.47, 1.06)	
Missing	-----	
Level of Education		
≤9th grade	Referent	0.35
9-11th grade	1.57 (1.05, 2.35)	
High School Grad/GED or equivalent	1.31 (0.77, 2.20)	
Some College or AA degree	1.32 (0.85, 2.05)	
College Graduate or Above	1.37 (0.81, 2.34)	
Missing	-----	
Smoking (Someone smokes at home)		
No	Referent	0.36
Yes	1.21 (0.78, 1.89)	
Missing	0.38 (0.49, 2.97)	

NHANES Measure	Odds Ratio (95% CI) (weighted)	p-value (Pearson)
Alcohol Consumption (Number of alcoholic drinks in the past month)		
≤1	Referent	0.21
2	0.55 (0.23, 1.30)	
≥3 & ≤40	0.52 (0.24, 1.12)	
Missing	0.51 (0.28, 0.93)	
Stomach or Intestinal Illness (with vomiting and diarrhea in the past 30 days)		
No	Referent	0.10
Yes	2.02 (1.08, 3.78)	
Missing	1.2 (0.77, 1.87)	
Income		
$0-9,999	Referent	<0.00
$10,000-14,999	3.52 (1.70, 7.3)	
$15,000-19,999	2.06 (0.56, 7.49)	
$20,000-24,999	2.54 (1.31, 4.94)	
$25,000-34,999	1.28 (0.59, 2.79)	
$35,000-44,999	2.79 (1.39, 5.58)	
$45,000-54,999	1.80 (0.89, 3.63)	
$55,000-74,999	2.65 (1.15, 6.13)	
≥$75,000	2.15 (0.90, 5.15)	
Missing	2.76 (1.71, 4.47)	
Veteran/Military Status		
No	Referent	0.32
Yes	1.37 (0.71, 2.65)	
Missing	------	
People per Household		
1	Referent	0.80
2	0.63 (0.31, 1.26)	
3	0.63 (0.31, 1.25)	
4	0.60 (0.30, 1.18)	
5	0.89 (0.30, 2.63)	
6	0.76 (0.25, 2.29)	
7	0.72 (0.20, 2.63)	
Missing	------	

Table 10: Confounder assessment using sample weights, NHANES 1999-2000.

NHANES Measure	Crude OR	Adjusted OR	Percent Difference ((Crude-Adjusted/Crude)*100)
Lifetime Peptic Ulcers	-------	------	------
RBC Folic Acid (ng/ml)	0.94	------	------
	0.84	------	------
	0.94	------	------
H. pylori (ELISA Immune Status Ratio (ISR))	0.94	0.93	0.25%
	0.84	0.84	0.31%*
	0.94	0.92	2.41%
Gender	0.94	0.95	-1.10%
	0.84	0.84	0.74%*
	0.94	0.95	-0.91%
Marital Status	0.94	0.93	0.39%
	0.84	0.84	0.26%*
	0.94	0.94	0.11%*
Ethnicity	0.94	0.94	0.26%*
	0.84	0.83	0.05%
	0.94	0.94	0.09%*
Age (years)	0.94	0.93	0.38%
	0.84	0.83	0.38%
	0.94	0.94	0.11%*
Level of Education	0.94	0.94	0.00%*
	0.84	0.84	0.23%*
	0.94	0.94	0.02%*
Smoking (Someone smokes at home)	0.94	0.94	0.00%
	0.84	0.84	0.21%*
	0.94	0.94	0.27%*
Stomach or Intestinal Illness (with vomiting and diarrhea in the past 30 days)	0.94	0.94	0.22%*
	0.84	0.83	0.16%
	0.94	0.89	5.01%
Income	0.94	0.94	0.36%*
	0.84	0.84	0.14%*
	0.94	0.94	0.25%*

NHANES Measure	Crude OR	Adjusted OR	Percent Difference ((Crude-Adjusted/Crude)*100)
Veteran/Military Status	0.94	0.92	1.51%
	0.84	0.83	0.76%
	0.94	0.93	1.10%
People per Household	0.94	0.94	0.06%*
	0.84	0.83	0.26%
	0.94	0.94	0.15%*

*Percent difference not equal to 0 due to rounding.

Table 11: Step-by-step summary of multiple logistic regression model, odds ratio, and p-values using sample weights, NHANES 1999-2000.

Characteristics	Stage 1 OR, (p-value)	Stage 2 OR, (p-value)	Stage 3 OR, (p-value)	Stage 4 OR, (p-value)	Stage 5 OR, (p-value)	Stage 6 OR, (p-value)	Stage 7 OR, (p-value)
Lifetime Peptic Ulcers	----	----	----	----	----	----	----
RBC folic acid (ng/ml)	----	----	----	----	----	----	----
≤ 227	Referent	Referent	Referent	Referent	Referent	Referent	Referent
≥228 & ≤301	0.93(0.82)	0.93 (0.81)	0.92 (0.79)	0.94 (0.82)	0.93 (0.81)	0.94 (0.82)	0.943(0.81)
≥302 & ≤628	0.84(0.57)	0.84 (0.57)	0.85 (0.60)	0.85 (0.62)	0.85 (0.63)	0.85 (0.62)	0.85 (0.62)
≥629	0.94(0.78)	0.92 (0.71	0.93 (0.77)	0.94 (0.80)	0.94 (0.81)	0.95 (0.81)	0.94 (0.81)
H. pylori (ELISA Immune Status Ratio (ISR))		1.06 (0.47)	1.06 (0.47)	1.06 (0.47)	1.06 (0.49)	1.06 (0.48)	1.06 (0.46)
NSAIDs (Products taken)			1.00 (0.14)	1.00 (0.12)	1.00 (0.12)	1.00 (0.12)	1.00 (0.13)
Gender				1.10 (0.45)	1.10 (0.43)	1.10 (0.44)	1.12 (0.38)
Marital Status					1.00 (0.35)	1.00 (0.34)	1.00 (0.32)
Ethnicity						0.97 (0.79)	0.97 (0.83)
Age (years)							0.90 (0.07)

Characteristics	Stage 8 OR, (p-value)	Stage 9 OR, (p-value)	Stage 10 OR, (p-value)	Stage 11 OR, (p-value)	Stage 12 OR, (p-value)	Stage 13 OR, (p-value)	Stage 14 OR, (p-value)
Lifetime Peptic Ulcers	----	----	----	----	----	----	----
RBC folic acid (ng/ml)	----	----	----	----	----	----	----
≤ 227	Referent	Referent	Referent	Referent	Referent	Referent	Referent
≥228 & ≤301	0.93 (0.81)	0.93 (0.82)	0.94 (8.43)	0.94 (0.84)	0.94 (0.85)	0.94 (0.83)	0.94 (0.83)
≥302 & ≤628	0.85 (0.63)	0.86 (0.64)	0.86 (0.64)	0.86 (0.64)	0.86 (0.64)	0.86 (0.64)	0.85 (0.62)
≥629	0.94 (0.80)	0.94 (0.79)	0.96 (0.87)	0.90 (0.74)	0.89 (0.72)	0.89 (0.71)	0.89 (0.72)
H. pylori (ELISA Immune Status Ratio (ISR))	1.06 (0.46)	1.07 (0.44)	1.07 (0.40)	1.07 (0.42)	1.07 (0.41)	1.07 (0.41)	1.07 (0.40)
NSAIDs (Products taken)	1.00 (0.13)	1.00 (0.13)	1.00 (0.28)	1.00 (0.27)	1.00 (0.30)	1.00 (0.29)	1.00 (0.31)
Gender	1.12 (0.38)	1.11 (0.41)	1.11 (0.42)	1.12 (0.41)	1.11 (0.45)	1.28 (0.19)	1.28 (0.20)
Marital Status	1.00 (0.32)	1.00 (0.35)	1.00 (0.36)	1.00 (0.37)	1.00 (0.34)	1.00 (0.33)	1.00 (0.33)
Ethnicity	0.98 (0.84)	0.98 (0.84)	0.98 (0.84)	0.97 (0.83)	0.97 (0.83)	0.97 (0.76)	0.95 (0.70)
Age (years)	0.90 (0.07)	0.90 (0.07)	0.90 (0.06)	0.90 (0.06)	0.89 (0.06)	0.86 (0.03)	0.85 (0.02)
Level of Education	0.99 (0.50)	0.99 (0.56)	0.99 (0.64)	0.99 (0.73)	1.00 (0.47)	0.99 (0.78)	0.99 (0.89)
Smoking (Someone smokes at home)		1.00 (0.28)	1.00 (0.30)	1.00 (0.30)	1.00 (0.21)	1.00 (0.24)	1.00 (0.25)
Alcohol Consumption (Number of alcoholic drinks in the past month)			1.00 (0.30)	1.00 (0.30)	1.00 (0.29)	1.00 (0.33)	1.00 (0.33)
Stomach or Intestinal Illness (with vomiting and diarrhea in the past 30 days)				1.00 (0.60)	1.00 (0.55)	1.00 (0.55)	1.00 (0.56)
Income					1.00 (0.12)	1.00 (0.12)	1.00 (0.10)
Veteran/Military Status						1.64 (0.22)	0.16 (0.23)
People per Household							0.96 (0.57)

Association:

We used multivariate logistic regression to determine the association between folic acid blood levels and the history of ulcers within a lifetime (Table 12). The model showed that as compared to the baseline range of 0ng/ml to 227ng/ml, the odds of ever having peptic ulcers remain mostly the same at the 228ng/ml to 301ng/ml range (OR= 1.00; 95% CI: 0.54,1.87), the odds of developing peptic ulcers decreases at the 302ng/ml to 628ng/ml range (OR=0.79; 95% CI: 0.40,1.58), and the odds of ever having peptic ulcers also decreases above 629ng/ml (OR=0.82; 95% CI: 0.44,1.55). The overall association, after accounting for all other variables, was not statistically significant (p=0.86). Even though the values for the odds ratios were not statistically significant, the overall model had an adequate fit above the required statistical significance (p=0.33; weighted goodness of fit) (Appendix IV). A test for trend of the association showed no statistical significance for a dose response effect of decreasing odds of lifetime peptic ulcers with increasing RBC folic acid (p=0.393).

Other M*ultivariate Logistic Regression Results*

Our multivariate logistic regression analysis demonstrated that all 11 confounding variables that we included in the final mode were not statistically significant, except for income. We included *H.*pylori in our final statistical model. Our results show that as compared to baseline ISR of equal or less than 0.2, the odds of ever having a lifetime peptic ulcers is reduced at above 0.2 to 0.38 ISR (OR= 0.78; 95% CI: 0.48,1.28), the odds of ever having a lifetime peptic

ulcers is reduced at above 0.38 to 1.72 ISR (OR=0.52; CI: 0.29,0.94), and the odds of every

having a lifetime peptic ulcer is increased at above 1.72 ISR (OR=1.15; CI:0.76,1.76). The

association between *H.pylori* and the main outcome variable was not statistically significant

(p=0.18) (table 12).

We included gender in our final statistical model. Our results show that as compared to

males, the odds of developing a lifetime peptic ulcer as a female increase (OR=1.25;

CI:0.83,1.89). The association between gender and the main outcome variable was not

statistically significant (p=0.25) (table 12).

We included marital status in our final statistical model. Our results show that as

compared to a married person, the odds of developing a lifetime peptic ulcer as widowed

increases (OR=1.16; CI:0.54,2.48), the odds of developing a lifetime peptic ulcer as divorced

increase (OR=1.15; CI:0.47, 2.82), the odds of developing a lifetime peptic ulcer as separated

increase slightly (OR= 1.01; CI:0.28,3.61), the odds of developing a lifetime peptic ulcer as never

married decreases (OR= 0.95; CI:0.63,1.42), the odds of developing a lifetime peptic ulcer as

living with a partner increases (OR=1.21; CI:0.47,3.16), and the odds of developing a lifetime

peptic ulcer for those who responded to the main outcome variable question and not to the

question pertaining to this confounder increased (OR=1.24; CI:0.84,1.82). The association

between marital status and the main outcome variable was not statistically significant (p=0.80)

(table 12).

We included ethnicity in our final statistical model. Our results show that as compared

to a person of Mexican American descent, the odds of developing a lifetime peptic ulcer for

people of Other Hispanic descent increases (1.85; CI:1.00,3.46), the odds of developing a lifetime peptic ulcer for people of Non-Hispanic White descent increases (1.08; CI:0.62,1.89), the odds of developing a lifetime peptic ulcer for people of Non-Hispanic Black descent increases (OR=1.52; CI:0.92, 2.51), and the odds of developing a lifetime peptic ulcer for people of other descent decreases (OR=0.88; CI:0.19, 4.00). The association between ethnicity and the main outcome variable was not statistically significant (p=0.25) (table 12).

We included age (years) in our final statistical model. Our results show that as compared to a person of age between 20 years and 34 years, the odds of developing a lifetime peptic ulcer for people of ages 35 years to 49 years decreases (OR=0.72; CI:0.35,1.50), the odds of developing a lifetime peptic ulcer for people of ages 50 years to 67 years decreases (OR=0.75; CI:0.45,1.26), and the odds of developing a lifetime peptic ulcer for people of age 68 years and above decreases (OR=0.62; CI:0.43, 0.90). The association between age (years) and the main outcome variable was not statistically significant (p=0.12) (table 12).

We included level of education in our final statistical model. Our results show that as compared to someone with a 9th grade education or less, the odds of developing a lifetime peptic ulcer for someone with an education between 9th and 11th grade increases (OR=1.41; CI:0.86,2.32), the odds of developing a lifetime peptic ulcer for someone with a High School Grad/GED or equivalent education increases (OR=1.21, CI:0.66,2.22), the odds of developing a lifetime peptic ulcer for someone with some College or AA degree increases (OR=1.19; CI:0.65,2.20), the odds of developing a lifetime peptic ulcer for a College Graduate or above

increases (OR=1.26; CI:0.8,1.99). The association between level of education and the main outcome variable was not statistically significant (p=0.68) (table 12).

We included smoking (someone smokes at home) in our final statistical model. Our results show that as compared to people non exposed to smoking, those who are exposed increase the odds of developing a lifetime peptic ulcer (OR=1.31; CI:0.82,2.09), and those who responded to the question regarding the main outcome variable and did not respond to the question pertaining this confounding variable decreased the odds of developing a lifetime peptic ulcer (OR=0.34; CI:0.04, 3.15). The association between smoking (someone smokes at home) and the main outcome variable was not statistically significant (p=0.37) (table 12).

We included stomach or intestinal illness (with vomiting and diarrhea in the past 30 days) in our final statistical model. Our results show that as compared to those who reported yes, the ones who reported no increased the odds of developing a lifetime peptic ulcer (OR=2.01; CI:1.09,3.70), and the ones who answered to the question regarding the main outcome variable and did not answer to the question pertaining this confounding variable increased the odds of developing a lifetime peptic ulcer (OR=1.16; CI:0.62,2.18). The association between stomach or intestinal illness (with vomiting and diarrhea in the past 30 days) was not statistically significant (p=0.09) (table 12).

We included income in our final statistical model. Our results show that as compared to an income between $0 and $9,999, the odds of developing a lifetime peptic ulcer increases for an income between $10,000 and $14,999 (OR3.89; CI:1.81,8.37), the odds of developing a lifetime peptic ulcer increases for an income between $15,000 and $19,999 (OR=2.43;

54

CI:1.27,4.64), the odds of developing a lifetime peptic ulcer increases with an income between $20,000 and $24,999 (OR=1.27; CI:0.55,2.93), the odds of developing a lifetime peptic ulcer increases with an income between $25,000 and $34,999 (OR=2.96; CI:1.36,6.41), the odds of developing a lifetime peptic ulcer increases with an income between $35,000 and $44,999 (OR=1.73; CI:0.74,4.00), the odds of developing a lifetime peptic ulcer increases with an income between $45,000 and $54,999 (OR=2.72; CI:1.15,6.43), the odds of developing a lifetime peptic ulcer increases with an income between $55,000 to $$74,999 (OR=2.26; CI:0.91,5.58), and the odds of developing a lifetime peptic ulcer increases with an income at or above $75,000. The association between income and the main outcome variable was statistically significant (p<0.00).

We included veteran/marital status in the final statistical model. Our results show that as compared to those who are in a veteran/military status, the odds of developing a lifetime peptic ulcer for those who are not in a veteran/military status increases (OR1.69; CI:0.71,4.07). The association between veteran/military status and the main outcome variable was not statistically significant (p=0.22) (table 12).

Table 12: Multivariate logistic regression association between lifetime history of peptic ulcers and RBC folic acid levels using sample weights, NHANES 1999-2000.

NHANES Measure	Odds Ratio (95% CI)	p-value
RBC Folic Acid (ng/ml)		
≤ 227	Referent	0.86
≥228 & ≤301	1.00 (0.54, 1.87)	
≥302 & ≤628	0.79 (0.40, 1.58)	
≥629	0.82 (0.44, 1.55)	
H. pylori **(ELISA Immune Status Ratio (ISR))**		
≤ 0.2	Referent	0.18
>0.2 & ≤0.38	0.78 (0.48, 1.28)	
>0.38 & ≤1.72	0.52 (0.29, 0.94)	
>1.72	1.15 (0.76, 1.76)	
Gender		
Male	Referent	0.25
Female	1.25 (0.83, 1.89)	
Marital Status		
Married	Referent	0.80
Widowed	1.16 (0.54, 2.48)	
Divorced	1.15 (0.47, 2.82)	
Separated	1.01 (0.28, 3.61)	
Never married	0.95 (0.63, 1.42)	
Living with partner	1.21 (0.47, 3.16)	
Missing	1.24 (0.84, 1.82)	
Ethnicity		
Mexican American	Referent	0.25
Other Hispanic	1.85 (1.00, 3.46)	
Non-Hispanic White	1.08 (0.62, 1.89)	
Non-Hispanic Black	1.52 (0.92, 2.51)	
Other	0.88 (0.19, 4.00)	
Age (years)		
≥20 & ≤34	Referent	0.12
≥35 & ≤49	0.72 (0.35, 1.50)	
≥50 & ≤67	0.75 (0.45, 1.26)	
≥68	0.62 (0.43, 0.90)	

NHANES Measure	Odds Ratio (95% CI)	p-value
Level of Education		
≤9th grade	Referent	0.68
9-11th grade	1.41 (0.86, 2.32)	
High School Grad/GED or equivalent	1.21 (0.66, 2.22)	
Some College or AA degree	1.19 (0.65, 2.20)	
College Graduate or Above	1.26 (0.80, 1.99)	
Smoking (Someone smokes at home)		
No	Referent	0.37
Yes	1.31 (0.82, 2.09)	
Missing	0.34 (0.04, 3.15)	
Stomach or intestinal illness (with vomiting and diarrhea in the past 30 days)		
Yes	Referent	0.09
No	2.01 (1.09, 3.70)	
Missing	1.16 (0.62, 2.18)	
Income		
$0-9,999	Referent	<0.00
$10,000-14,999	3.89 (1.81, 8.37)	
$15,000-19,999	2.43 (1.27, 4.64)	
$20,000-24,999	1.27 (0.55, 2.93)	
$25,000-34,999	2.96 (1.36, 6.41)	
$35,000-44,999	1.73 (0.74, 4.00)	
$45,000-54,999	2.72 (1.15, 6.43)	
$55,000-74,999	2.26 (0.91, 5.58)	
≥$75,000	3.11 (1.83, 5.30)	
Veteran/Military Status		
Yes	Referent	0.22
No	1.69 (0.71, 4.07)	

DISCUSSION

The results of this cross sectional study with data from the NHANES 1999-2000 database do not support convincingly an association between folic acid levels and history of peptic ulcers. Nonetheless, a protective trend is observed as the levels of RBC folic acid increase.

Strengths

This study has several strengths. One is the novelty of being first in evaluating an association between RBC folic acid and history of peptic ulcers. No other study has explored this association before. In addition, this study did analyze the NHANES 1999-2000 dataset, a reliable and validated governmental database. The survey is carefully designed to maintain the appropriate level of precision for racial and ethnic subgroups, while assuring that the data are representative of the entire nation [59]. In theory each participant represents 50,000 other residents of the U.S. [60]. People age 20 or younger were excluded, since young people are less likely to have peptic ulcers than older people, and the results should be more accurate. Patients, providers, and researchers will benefit from the information produced by this study.

Limitations

On the other hand, several weaknesses exist in this study. First, this is a cross-sectional study that takes a snapshot of the population, and temporality is difficult to establish.

Temporality is a necessary piece in establishing causality, and to determine the direction of the association. It is imaginable that RBC folic acid would have an association with peptic ulcer, or that possibly peptic ulcer causes people to increase their levels of RBC folic acid. However, several approaches were used in attempting to overcome this weakness. First, RBC folic acid is the best indicator of long-term folate stores because the average life span of a red blood cell has been estimated to be 120 days[61]. Also, peptic ulcers were based on events within a lifetime. Therefore, the type of method used by NHANES in collecting data for the dependent and independent variables helps somewhat to alleviate the controversy related to temporality that comes about in a cross sectional study. However, the time spam for a peptic ulcer event within a lifetime is longer than the lifespan of a red blood cell, and therefore limitations regarding temporality cannot be eliminated. (Apendix II).

An inadequate sample size was difficult to work with. Limited data impaired determining an association between peptic ulcers within a year, unlike peptic ulcers within a lifetime. Assessing for peptic ulcers within a year would have rendered a more accurate association with RBC folic acid levels, but assessing for history of lifetime peptic ulcers was also valid. Even though history of lifetime peptic ulcers has a longer timeframe than RBC folic acid, the association between these two variables is better than serum folic acid.

Three of the originally selected confounding variables were discarded. Those of main concern are NSAID use and alcohol consumption. Unfortunately the data provided by the NHANES 1999-2000 was not sufficient in numbers to include these variables, which is something worthy of consideration for future studies using this data. Not accounting for those

variables could mean that the results of this study are confounded. Moreover, discarding the variable NSAIDs consumption impaired the assessment of some of the specific aims. Caution should be considered when using this secondary data source.

The variable smoking refers to passive smokers. The question pertaining to this variable asked "does anyone smokes in the home. These individuals are exposed to tobacco or cigarette smoke, but do not actively participate. Regardless of the type of exposure, cigarette smoke has been cited in the literature as a factor leading to risks of developing peptic ulcers. It leads to gastric lesion formation [62].

Most of the results of the study were not statistically significant. Carrying out a confounding variable selection was difficult because our statistical analyses showed that the association between the main outcome variable and the main predictor variable did not change much. Fortunately, enough information was provided in the literature to overcome this shortcoming, and the necessary variables were selected to build the final statistical model. The results on the association between folic acid levels and peptic ulcers within a lifetime are also not statistically significant; however, a trend is observed in risk reduction of peptic ulcer within a lifetime as the blood levels of folic acid increase.

The prevalence of peptic ulcers identified in this study is also higher than the one reported in other studies[17,18]. In this study a prevalence of lifetime peptic ulcers was found to be 9.9%. While the prevalence of peptic ulcers within a year was reported by the study of Sonnenberg and colleagues as 3.35% (2.56% with diagnostic confirmation), it was also reported to be 10.28% (7.71% with diagnostic confirmation) within any time in the past[17]. The latter

prevalence is aligned with the prevalence found in this study. Since this study focused on lifetime peptic ulcers, as compared to ulcers within a year, it is expected that the prevalence would be higher.

CONCLUSION

The results of the study are not statistically significant. Therefore, it becomes difficult to determine statistically, that an association truly exists between lifetime peptic ulcers and blood levels of folic acid. Also, it is statistically difficult to determine an existing trend in risk reduction as the levels of blood folic acid increase. Future studies might consider the results of this study to establish the benefits of folic acid consumption in relation to a reduction in outcomes of lifetime peptic ulcer. However, they should be cautious about the use of the NHANES 1999-2000 database.

It is biologically plausible that an association exists between folic acid consumption and a reduction in occurrence of peptic ulcers. Folic acid helps with the synthesis of DNA and amino acids. Therefore, it is plausible to think that folic acid helps restore cells affected through ulcerations in the gastrointestinal tract.

This study explored the possibility that an association exists between RBC folic acid levels and a reduction in risk of lifetime outcomes of peptic ulcers, which provides the theoretical framework and foundation for future studies to confirm a true association between these two factors.

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Appendix I

RBC Folate, Serum Folate, and Vitamin B12

Both serum folate and vitamin B12 are measured by using the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay kit.[5] The assay is performed by combining serum or a whole blood hemolysate sample with ^{125}I-folate and ^{57}Co-vitamin B12 in a solution containing dithiothreitol (DTT) and cyanide. The mixture is boiled to inactivate

endogenous folate-binding proteins and to convert the various forms of vitamin B12 to cyanocobalamin. The reduced folate and its analogs are stabilized by DTT during the heating. The mixture is cooled and then combined with immobilized affinity-purified porcine intrinsic factor and folate-binding proteins. The addition of these substances adjusts and buffers the pH of the reaction mixture to 9.2. The reaction mixture is then incubated for 1 hour at room temperature.

During incubation, the endogenous and labeled folate and B12 compete for the limited number of binding sites on the basis of their relative concentrations. The reaction mixtures are then centrifuged and decanted. Labeled and unlabeled folate and vitamin B12, binding to immobilized binding proteins, are concentrated in the bottom of the tube in the form of a pellet. The unbound folate and B12 in the supernatant are discarded, and the radioactivity associated with the pellet is counted. Standard curves are prepared by using the pre-calibrated folate/B12 standards in a human serum albumin base. The concentration of the folate and vitamin B12 in the participant's serum or folate in a participant's whole blood is calculated from the standard curve.

In the erythrocyte folate procedure, the sample is first diluted 1:11 with a solution of 1 g/dL ascorbic acid in water and either incubated for 90 min prior to assay or frozen immediately for later assay. The 90-minute incubation or the freeze-thaw is necessary for hemolysis of the red blood cells; either allows the endogenous folate conjugates to hydrolyze the conjugated pterylpolyglutamates prior to assay. The sample is further diluted 1:2 with a protein diluent (human serum albumin), resulting in a matrix similar to that of the standards and serum samples.

Data Access

All data are publicly available.

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Description

C-reactive protein
C-reactive protein is considered one of the best measures of the acute phase response to an infectious disease or other cause of tissue damage and inflammation. It is used to correct the iron status measures, which are affected by inflammation. It can also be used to measure the body's response to inflammation from chronic conditions, such as arthritis, and environmental exposures to agents such as tobacco smoke.

Helicobacter pylori
This organism has been shown to be the causative agent in chronic-active gastritis, and evidence has almost completely satisfied Koch's postulates for this organisms' pathogenicity in primary duodenal ulcers. More recent evidence has suggested that chronic H. pylori infection as well as early age of H. pylori- acquisition is a critical precursor to gastric carcinoma. Although an explosion of research has occurred over the past decade, many fundamental questions on the route of transmission and the role of environmental risk factors (i.e. food, water) remain to be answered. Because NHANES will have numerous data on environmental exposures in addition to demographic data on participants, these data can be analyzed to add information on potential route of transmission for this organism.

Analytic Methodology

C-reactive protein
This method quantifies C-reactive protein (CRP) by latex-enhanced nephelometry. Particle-enhanced assays are based on the reaction between a soluble analyte and the corresponding antigen or antibody bound to polystyrene particles. For the quantification of CRP, particles consisting of a polystyrene core and a hydrophilic shell are used in order to link anti-CRP antibodies covalently.
A dilute solution of test sample is mixed with latex particles coated with mouse monoclonal anti-CRP antibodies. CRP present in the test sample will form an antigen-antibody complex with the latex particles.

An automatic blank subtraction is performed. CRP concentrations are calculated by using a calibration curve. Data reduction of the signals is performed by using a storable logit-log function for the calibration curve. These assays are performed on a Behring Nephelometer for quantitative CRP determination.

Helicobacter pylori

The Wampole Laboratories (Wampole) H. pylori IgG Enzyme-Linked Immunosorbent Assays (ELISA) is intended for the detection and qualitative determination of IgG antibodies to Helicobacter pylori in human serum.

Enzyme linked immunosorbent assays (ELISA) rely on the ability of biological materials, (i.e. antigens) to adsorb to plastic surfaces such as polystyrene (solid phase). When antigens bound to the solid phase are brought into contact with a participant's serum, antigen-specific antibody, if present, will bind to the antigen on the solid phase forming antigen-antibody complexes. Excess antibody is removed by washing. This is followed by the addition of goat anti-human IgG conjugated with horseradish peroxidase, which then binds to the antibody-antigen complexes. The excess conjugate is removed by washing, followed by the addition of chromogen substrate tetramethylbenzidine (TMB). If specific antibody to the antigen is present in the participant's serum, a blue color develops. When the enzymatic reaction is stopped with 1N H_2SO_4, the contentsof the wells turn yellow. The color, which is proportional to the concentration of antibody in the serum, can be read on a suitable spectrophotometer or ELISA microwell plate reader. The sensitivity, specificity, and reproducibility of enzyme- linked immunoassays can be comparable to other serological tests for antibody, such as immunofluorescence, complement fixation, hemagglutination, and radioimmunoassays.

LBXHP1

A value <0.90 is considered negative for the presence of detectable IgG antibody. Values between 0.91-1.09 are considered equivocal and values greater than 1.10 indicates the presence of detectable IgG antibody.

Medical Conditions (MCQ) Section (June 2002)
Sample Person Questionnaire

Description

The medical conditions section (prefix **MCQ**) provides self-reported personal interview data on a broad range of health conditions for both children and adults. Many questions in the NHANES 1999-2000 MCQ section are also contained in previous NHANES surveys allowing researchers to monitor health trends over time. The NHANES MCQ questionnaire segment is generally modeled on the "Medical Conditions" questionnaire section of the U.S. National Health Interview Survey. In accordance with the Department of Health and Human Services' Survey Integration Plan, a number of questions on health conditions are in fact identical to those used in the redesigned National Health Interview Survey adult and child core sections that started in 1997.

Major Topics in the MCQ Section

Major health conditions:

Asthma, Childhood and Adult	Emphysema
Anemia	Goiter, Other Thyroid disease
Angina	Hay Fever
Attention Deficit Disorder (ADD)	Headache
Arthritis (Rheumatoid & Osteoarthritis)	Heart Attack (Myocardial Infarction)
Cancer (Multiple Varieties)	Learning Disability
Chickenpox (Varicella)	Liver Disease
Chronic Bronchitis	Overweight
Congestive Heart Failure	Peptic Ulcer
Coronary Heart Disease	Stroke
Ear Infections	

Diet Behavior and Nutrition Section (June 2002)
Sample Person Questionnaire

Description of Section

The Diet Behavior and Nutrition (DBQ) section provides personal interview data on several nutrition topics. Many of the questions in the NHANES 1999 DBQ section were included in NHANES II, 1976-80, Hispanic HANES 1982-84, and NHANES III, 1988-94. There are different target age groups for the topics in this section. For example, the questions pertaining to infant nutrition and breast-feeding were asked of proxy respondents for children 6 years of age and younger; alcohol consumption frequency questions were asked of persons 20+ years of age; and senior meal program participation questions were asked of respondents 60+ years of age. Data users should review the survey questionnaire thoroughly to determine the appropriate population targeted for each topic.

Topics and the target population groups in the DBQ section:

- Breastfeeding and other childhood feeding practices ($\leq$ 6 yr)
- Restaurant meal frequency ($\geq$1 yr)
- Table salt use and salt type used ($\geq$1 yr)
- Dark green vegetables and dried beans or peas consumption frequency ($\geq$2 yr)
- Poultry consumption (yes/no) and poultry skin trim practices ($\geq$12 yr)
- Meat consumption (yes/no) and meat fat trim practices ($\geq$12 yr)
- Current milk consumption pattern and types of milk ($\geq$1 yr)
- Milk consumption pattern throughout the life span ($\geq$20 yr)
- Alcohol consumption frequency by type of alcohol ($\geq$20 yr)
- Food consumption practices for elderly ($\geq$60 yr):
    - Screener for servings of 5 major food groups
    - Home-delivered meals in the past 12 months
    - Meals eaten at community feeding site in the past 12 months
- School meal program participation (4-19 yr)

Eligible Sample and Exclusion Criteria

The target age groups for questions in this section vary. Please review the questionnaire and codebook carefully.

Data Processing and Editing

Edit decisions were made to ensure the completeness, consistency, and analytic usefulness of the data. Systematic data editing was conducted to:

Standardize the frequency of alcohol consumption reported to number of times per month (ALD240, ALD250, and ALD260). These variables were derived from the two-part (number and unit) questions ALQ240, ALQ250, and ALQ260, using the conversion factors 7 days/week and 30.4 days/month. If the frequency was reported as "never," the value was recorded as zero. It is important to note that the portion sizes were not defined, and responses represent "number of times" as determined by the respondent.

Special Notes on Using the Dataset

In this section, frequency of alcohol consumption during the past 30 days was collected for participants aged 20 years and older by alcohol type. There is additional information on alcohol use in the Survey, including: 1) 24-hour dietary recall data on alcohol consumption which can be used to quantify the contribution of alcohol to total food energy intake and produce population reference data on alcohol intake; 2) MEC interview data on quantifiable current alcohol use and life time drinking behavior for adults aged 20 years and above; and 3) MEC Audio-CASI data on alcohol use in adolescents aged 12-19 years old. Please refer to the documentation and codebooks for these sections for data analysis.

Analgesic Medications Subsection (June 2002)
Dietary Supplements and Prescription Medication Section
Household Interview-Sample Person Questionnaire

Description

The Analgesic Subsection (RXQ_ANA) is part of the Dietary Supplements and Prescription Medication Section of the Sample Person Questionnaire.

Data on lifetime and current, chronic use of specific prescription and nonprescription analgesic medications were collected.

Eligible Sample

Survey participants 20 years of age and older

Data Collection

Respondents were asked a series of questions about lifetime and current chronic use of specific prescription and nonprescription pain medication that were identified on a hand card shown to the participant by the interviewer (refer below). Chronic use was defined as 'use nearly every day for as long as a month.'

A hand card listed the following pain-relieving products:

   Aspirin – also buffered aspirin products such as Anacin, Bayer, Bufferin, Midol, Ascripton, Ecotrin,
      Pabrin, and Alka Seltzer

   Tylenol – also other acetaminophen products, including sinus products such as Anacin-3, Dristan
      AF, and Comtrex

   Ibuprofen – also Advil, Nuprin, Motrin IB (including cold and sinus products containing ibuprofen)

         Excedrin
         Vanquish
         Feldene
         Voltarin
         Clinoril
         Indocin
         Naprosyn – also Aleve
         Tolectin

Data Editing

For the question on the number of years of use of an analgesic product nearly every day, some participants reported use for more than one year, however, they did not provide the exact number of years of use. The derived variable (RXD320) on this data release file records the responses for these individuals as "used for more than one year, unspecified."

Analytic Notes

This file is a drug product level file. Therefore, participants who reported the use of multiple analgesics will have a record for each reported analgesic.

Note that the data do not differentiate between prescription and nonprescription analgesics.

Composite International Diagnostic Interview (Generalized Anxiety Disorder- CIQGAD, Major Depression Module-CIQMDEP, Panic Disorder Module-CIQPANIC)

Component Description

An NHANES version of three modules from the automated version of the World Health Organization Composite International Diagnostic Interview, Version 2.1 (CIDI-Auto 2.1) was administered during the face-to-face portion of the Mobile Examination Center (MEC) interview. The CIDI is a comprehensive, fully standardized interview that is used to assess mental disorders and provide diagnoses according to definitions and criteria of the tenth revision of the International Classification of Diseases (ICD-10, World Health Organization 1992, 1993) and the fourth edition of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, 1994). The CIDI is especially suitable for large epidemiological studies because it can be administered by lay interviewers, does not require outside informants or medical records, and does not assume the presence of a current disorder. It is used in both clinical and research settings.

The NHANES CIDI, developed as a computer-administered version, consisted of three diagnostic modules that addressed diagnoses present in the past 12 months. These modules were Panic Disorder, Generalized Anxiety Disorder, and Depressive Disorders. Additional questions were added to measure both the quality and quantity of impairment, and to assess risk factors such as physical illness and life events. These additional questions were placed at the end of each

module. The modules were administered in either English or Spanish by a trained interviewer who followed guidelines instituted by the CIDI Training Centers. These guidelines are included in the MEC Interviewer Manual, Chapter 6.

Eligible Sample

The instrument was administered to a half-sample of examination participants, ages 20 - 39 years. Proxies were ineligible, as were persons who required interpreters (i.e., non-English or Spanish speakers).

Interview Setting and Mode of Administration

Interview Setting

MEC private face-to-face interview.

Mode of Administration

In-person.

Data Processing and Editing

Questionnaire interview responses were entered by the interviewer on a desktop computer and then uploaded to the NHANES computerized database in the same manner as other sections of the MEC interview. In this public release data file, variables are included for responses to all questions to the instruments except for checkpoints and questions requiring text entries. All of the questions from the original instrument were renamed to be consistent with NHANES naming conventions. The coding patterns for the traditional CIDI response categories (1 = no, 5 = yes) were also modified. Both the original question names and the new NHANES names can be found in the corresponding codebook. NHANES CIDI modules can be distinguished by the pre-fix for the variable names as follows:

CIQPANIC: Generalized Panic Disorder
CIQGAD: Generalized Anxiety Disorder
CIQMDEP: Major Depressive Disorders

Analytic Notes

Special examination sample weights were developed for the half-sample that was eligible for the interview. Separate 2-year as well as 4-year (1999-2002) weights are included in the data file. Because of small samples sizes, it is recommended that the data from the 2-year files be combined and that the 4-year weights be used for all analyses.

Abbreviations frequently found in the codebooks and on variables labels:
WTA = worried, tense, or anxious
SED = sad, empty, or depressed
MDA = medication, drugs or alcohol

The NHANES CIDI questions were developed in accordance with the ICD-10 and DSM-IV criteria for three diagnoses. As part of the final data preparation effort, responses to the questions were evaluated using a computer algorithm, first to assess each criterion, and then to combine criteria into diagnoses. The diagnostic algorithms were constructed criterion by criterion, and all criteria had to be met for a diagnosis to be positive. If all of the criteria for a diagnosis were positive, the diagnosis was considered to be present for the past 12 months. The algorithms, written in SAS code, are found in the CIDI Appendices.

The "diagnostic score" variables are found at the end of each module. They are named as follows: CIDPSCOR for Panic, CIDGSCOR for GAD, and CIDGSCOR for Depression. A value of "1" indicates a positive diagnosis, and a value of "5" indicates a negative diagnosis.

APPENDIX II

<u>Laboratory Procedures and Quality Control of Folate/Vitamin B12</u>

1. Summary of Test Principle and Clinical Relevance

    a. Clinical relevance

Folic acid is required in cellular metabolism and hematopoiesis, and prolonged folic acid deficiency leads to megaloblastic anemia. Vitamin B12 is an essential cofactor in intermediary metabolism and is required for the biosynthesis of RNA and DNA. Since a deficiency of either vitamin may be the cause of megaloblastic anemia, it is essential to determine the levels of both vitamin B12 and folic acid to establish the etiology of the anemia. Untreated vitamin B12 deficiency may lead to severe anemia and potentially irreversible nervous system degeneration.

    b. Test principle

Both vitamins are measured by using the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay kit (1). The assay is performed by combining serum or a whole blood hemolysate sample with ^{125}I-folate and ^{57}Co-vitamin B12 in a solution containing dithiothreitol (DTT) and cyanide. The mixture is boiled to inactivate endogenous folate-binding proteins and to convert the various forms of vitamin B12 to cyanocobalamin. The reduced folate and its analogs are stabilized by DTT during the heating. The mixture is cooled and then combined with immobilized affinity-purified porcine intrinsic factor and folate-binding proteins. The addition of these substances adjusts and buffers the pH of the reaction mixture to 9.2. The reaction mixture is then incubated for 1 hour at room temperature.

During incubation, the endogenous and labeled folate and B12 compete for the limited number of binding sites on the basis of their relative concentrations. The reaction mixtures are then centrifuged and decanted. Labeled and unlabeled folate and vitamin B12, binding to immobilized binding proteins, are concentrated in the bottom of the tube in the form of a pellet. The unbound folate and B12 in the supernatant are discarded, and the radioactivity associated with the pellet is counted. Standard curves are prepared by using the pre-calibrated folate/B12 standards in a human serum albumin base. The concentration of the folate and vitamin B12 in the patient serum or folate in a patient's whole blood is calculated from the standard curve.

In the erythrocyte folate procedure, the sample is first diluted 1:11 with a solution of 1 g/dL ascorbic acid in water and either incubated for 90 min prior to assay or frozen immediately for later assay. The 90-min incubation or the freeze-thaw is necessary for hemolysis of the red blood cells; either allows the endogenous folate conjugates to hydrolyze the conjugated pterylpolyglutamates prior to assay. The sample is further diluted 1:2 with a protein diluent (human serum albumin), resulting in a matrix similar to that of the standards and serum samples.

2. Safety Precautions

The folate assay employs ^{125}I and ^{57}Co as tracers, and all necessary radiation safety considerations for isotope management and disposal must be observed according to the guidelines of the CDC Radiation Safety Manual. Any laboratory using radioimmunoassay (RIA) kits must hold a current NRC Certificate of Registration. In addition, all personnel must successfully complete the CDC training course, Radiation Safety in the Laboratory, or demonstrate equivalent instruction. All radioactive waste and contaminated material must be disposed of according to radiation safety guidelines.

Treat all serum specimens as potentially positive for infectious agents including HIV and hepatitis B. Observe Universal Precautions; wear protective gloves, labcoat, and safety glasses during all steps of this method because of both infectious and radioactive contamination hazards. We recommend the hepatitis B vaccine series for all analysts working with intact blood and serum sample materials. Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a labeled plastic autoclave bag for disposal.

Dithiothreitol, a primary reagent for this assay, is toxic. Avoid contact with eyes, skin, and clothing. Wash thoroughly after using. Wash immediately with plenty of water if exposed.

Material safety data sheets (MSDSs) for all chemicals contained in the kit are available in the MSDS section of the "Working Safely With Hazardous Chemicals" notebook located in the laboratory. MSDSs for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ohs.

3. Computerization; Data System Management

a. Calculation of serum folate (SFOL), vitamin B12, and whole blood folate (WBCF) values is accomplished with the software on the Packard Cobra gamma counter. SFOL, B12, and WBCF results are manually entered into a Microsoft Excel spreadsheet that calculates red blood cell folate (RBCF) based on the hematocrit. After a run is complete and any additional corrections by the analyst are made, the Excel result file (containing the patient data as well as the QC data) is electronically transferred to the appropriate analyte-specific subfolder in Q:/ITN/Nutrition Lab/Import into Access on the NCEH/DLS Local Area Network (LAN). The analyst also gives a hardcopy of the result file to the reviewing supervisor. After the reviewing supervisor approves the final values for release by checking off the bench and blind QC values and signing the hardcopy, he/she sends an email to the computer support staff that the data has been released to be imported into the NHANES 1999+ database that is located in Microsoft Access; the computer support staff imports the data into the NHANES 1999+ database by using a macro. Data entry is verified by the computer support staff and the supervisor. Data is transmitted electronically several times weekly to Westat's ISIS computer system, and transferred from there to NCHS. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.

b. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up once a week onto a floppy disk or a CD-ROM using a CD writer.

c. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

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4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

a. We recommend that specimen donors fast prior to specimen collection, but fasting is not required.

b. Serum folate and vitamin B12 assays are performed on fresh or frozen serum. RBC folate samples are prepared by diluting EDTA-whole blood 1:11 with 1 g/dL ascorbic acid and freezing the solution promptly, which keeps the folate in the reduced state.

c. A 400-µL serum sample is required for serum folate and vitamin B12 assays. A 400 µL solution consisting of a 100 µL whole blood specimen diluted with 1.0 mL of 1 g/dL ascorbic acid is necessary for the red cell folate assay. At assay time, 100 µL of this mixture is added to 100 µL of protein diluent in each of 2 tubes in order to provide the necessary final 1:22 dilution of the original sample for the red cell folate assay.

d. Serum specimens may be collected with regular red-top Vacutainers. Whole blood is collected with lavender-top Vacutainers that contain 1.5% K$_3$EDTA as an anticoagulant. A hematocrit measurement used for the red cell folate calculations is made at the time of collection. The appropriate amount of serum or whole blood/ascorbic acid solution is dispensed into a Nalge cryovial or other plastic screw-capped vial labeled with the participant's ID.

e. Specimens collected in the field should be frozen and then shipped on dry ice by overnight mail. Once received, they should be stored at –20 µC until analyzed. Serum folate and vitamin B12 are fairly stable if the serum is frozen at –20 to –70 µC before analysis. Ascorbic acid should not be added to the serum specimen because it will invalidate the B12 assay. Freeze-thaw cycles will cause degradation of the folate. Whole blood folate is especially sensitive to freeze-thaw degradation.

f. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site where the blood was collected. Some methods call for a 90-min incubation to hemolyze the red cells and allow the endogenous folate conjugates to hydrolyze the conjugated pteroylpolyglutamates to pteroylmonoglutamates prior to the assay for RBC folate. However, we have found that if the blood is diluted 1:11 with 1 g/dL ascorbic acid to keep the folate in the reduced state and the hemolysate is frozen promptly in the NHANES field vans, a single freeze-thaw cycle before analysis has the same effect as incubation (2).

g. Diurnal variation is not a major consideration. Hemolyzed serum specimens should not be used because they may have falsely high values. A recent article in Clinical Chemistry suggests that while serum vitamin B12 is light stable, serum folate specimens exposed to light for longer than 8 hours may have undergone 10-20% degradation (3). Therefore, specimens intended for folate analysis should be processed and stored frozen promptly if analysis is not to be performed within 8 hours of collection.

h. Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q:/ITN/Nutrition Laboratory/CLIA). The protocol discusses collection

and transport of specimens and the special equipment required. In general, plasma should be transported and stored at no more than –20°C. Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or plasma should be transferred into a sterile Nalge cryovial labelled with the participant's ID.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

    a. Reagent Preparation

(1) Working tracer reagent

Reconstitute the DTT with 10 mL deionized water. Agitate gently to dissolve, and let stand 5 min. Transfer the entire contents of the DTT vial into the appropriate tracer bottle. Cap and mix by inversion. Store at 2–8°C for 30 days.

(2) Red cell folate diluent

Add 5 mL deionized water to each vial needed. Allow to stand for 30 min. The solution will be stable for 1 month at –20°C. Two vials are required to prepare duplicate assay tubes for every 50 hemolysates.

(3) 1 g/dL Ascorbic acid solution

Add 1.0 g L-ascorbic to 100 mL deionized water and mix well to dissolve. Prepare fresh daily when needed for red cell hemolysates.

(4) Lyphochek levels I, II, III, and anemia control

Rehydrate each vial of Levels I-III with 5.0 mL deionized water and rehydrate the anemia control with 3.0 mL water. Mix the contents gently by swirling, and let stand for 15 min. Bio-Rad states that these quality control materials may be stored up to 10 days at 2–8°C. Our usual practice is to rehydrate and pool multiple vials of the same lot of a level, mix them well, aliquot 1.0 mL into polypropylene vials, and store them at –70°C to provide us with long-term quality control pools for our studies. One vial of each level is thawed for use on the day of analysis.

During the analysis of whole blood specimens, include Lyphochek red cell controls. Add 2 mL deionized water to each vial and treat the rehydrated contents as whole blood specimens and dilute them similarly for analysis. Again, we usually prepare and pool multiple vials of each level, dispense them as 1:11 hemolysates (1 part (100 _L) EDTA-whole blood with 10 parts 1% ascorbic acid (1.0 mL)), and store the vials at –70°C for long-term storage. The folate

concentrations in the materials vary from lot to lot, but they represent one deficient level, one normal-range level, and one elevated level (4).

(5) Additional higher concentration serum folate pool

Because occasional lots of the Bio-Rad Lyphochek materials do not exceed 10-11 ng/mL for serum folate concentration, and folate levels in the U.S. population are steadily increasing with supplementation and food fortification, and to ensure that pursuant to CLIA requirements we had a QC material in the higher concentration range (i.e., 11-20 ng/mL before dilution), we prepared an additional high concentration pool by collecting blood from pre-screened donors known to be supplementing. This blood was collected with anticoagulant and processed exactly as we stipulate for NHANES donors: it was allowed to clot for at least 30 minutes and no more than 60 minutes, then centrifuged and the serum was separated. Serum from multiple donors was combined and the final folate concentration was verified to be > 15 ng/mL but < 20 ng/mL (the highest standard concentration in the kit). One-mL aliquots were prepared from the filter-sterilized pooled material, and stored at −70°C. One aliquot is thawed and measured with each assay.

   b. Standards Preparation
Folate/Vitamin B12 Standards
These materials (0.0, 1.0, 2.5, 5.0, 10.0 and 20.0 ng/mL folate, and 0.0, 100, 250, 500, 1000, 2000 pg/mL vitamin B12) are supplied in a liquid form as pteroylglutamic acid (PGA) and cyanocobalamin in human serum albumin, ready to be used. If the entire kit is not used in one run, store the standards at 2–8°C until the expiration date of the kit. At pH 9.2 the binding affinities of PGA and N5-methyl-tetrahydrofolate (N5MeTHFA), the predominant biologically active form of monoglutamic folate in the body, are equivalent. PGA, however, is far more stable and can be used as an assay standard. It is also the standard material usually used in the traditional *Lactobacillus casei* microbiological assay for folate.

   c. Preparation of Quality Control Materials
As outlined previously, four levels of Lyphochek serum controls, and three red cell controls are analyzed in duplicate in each run as bench quality control materials. The controls are bought in bulk, rehydrated, mixed, re-aliquoted, and stored at −70°C. Approximate values are 1.0, 2.0, 6.0, 10.0 and 14.0 ng/mL for folate in serum; 60, 250, and 500 ng/mL in the red cell controls; and 100, 400, 700, 900 and 1600 pg/mL for vitamin B12. Bench QC pools may also be made from filter-sterilized fasting human serum that has been lyophilized or aliquoted in appropriate quantities and stored at −70°C.
For blind quality control pools, two levels (low-normal and high-normal ferritin concentrations) of blind QC pools may be prepared from pooled, filter-sterilized human serum obtained from fasting donors with elevated or decreased ferritin levels. Pool serum in acid-cleaned 20-L glass carboys. Mix well on a magnetic stirrer. Clean-filter the serum through in a sequential manner using filters of the following pore sizes, each preceded by a pre-filter: 3.00 μm, 1.20 μm, 0.80 μm, 0.65 μm, 0.45 μm, 0.30 μm, and 0.22 μm.

Through the use of sterile technique under a laminar-flow hood, dispense the serum in 1-mL aliquots with a Micromedic Digiflex dispenser into 2.0 mL Nalge cryovials. (A similar process, but without filter-sterilization, is used for the RBC folate samples.) Cap and label the vials with NHANES barcoded labels that have been specially prepared for the QC pools. Store the pools at −70°C at the CDC CASPIR Specimen Repository in Lawrenceville where they will be inserted randomly into the NHANES runs. Select 20 vials of each level for pool characterization

Other Materials (1) "Quantaphase II Folate or Folate/B12" radioassay kit (cat. no. 191-1046), 200-test size (Bio-Rad Laboratories).

(2) "Lyphochek" 3-level and "Lyphochek Anemia Control" lyophilized human serum quality control materials. Also "Lyphochek Red Cell Controls", levels I, II, and III (ECS Division, Bio-Rad Laboratories, Anaheim, CA).

(3) Disposable 12- x 75-mm polypropylene tubes (American Scientific Products, McGaw Park, IL).

(4) L-ascorbic acid, ACS certified (Fisher Scientific Co., Fairlawn, NJ).

(5) "FOAMRAC" foam rubber racks for holding tubes for decanting and blotting after centrifugation (Bio-Rad Laboratories).

(6) Red cell folate diluent: (human fraction V albumin solution) for diluting red cell hemolysates (Bio-Rad Laboratories).

(7) Combi-tips, 5.0- and 12.5-mL capacity (Brinkmann Instruments).

(8) Polypropylene test tube racks (Nalge Co., Rochester, NY).

    e. Instrumentation

(1) Packard Cobra gamma automatic gamma counter (Model E5005, Packard Instruments, Downers Grove, IL) or ICN Model 10/600 plus gamma counter (ICN Biomedical, Costa Mesa, CA).

(2) Model J6B centrifuge (Beckman Instruments, Inc., Palo Alto, CA), or Centra-7 centrifuge (International Equipment Co., Needham Heights, MA).

(3) Packard Multiprobe II Liquid Handling System (Packard Instruments, Downers Grove, IL)

(4) Multi-tube vortexer (Thermolyne Maximix III, VWR, Marietta, GA).

(5) Gilson Pipetman pipettor, 100- and 200 _L sizes (Rainin Instrument Co., Inc., Emeryville, CA).

(6) Eppendorf repeater pipettor (Brinkmann Instruments, Inc, Westbury, NY).

(7) Isotemp 220 water bath (Fisher Scientific, Norcross, GA).

7. Calibration and Calibration Verification Procedures

Results of in-house recovery studies using both forms of folate showed approximately 106% recovery for various levels of vitamin B12 added externally, 93% recovery for folate added to serum as N_5-methyl-tetrahydrofolate, and 99% recovery for PGA. External calibration may be verified with purified PGA; there is no National Institutes of Standards and Technology (NIST) standard reference material available for folate. The National Institute for Biological Specimens and Control (UK) has prepared an international vitamin B12 reference material, 320 pg/ampule, which will be used as an external B12 reference material at straight (320 pg/mL) and 1:2 dilution (160 pg/mL). The limits of detection as determined with dilutions of purified PGA and cyanocobalamin standards are 0.2 ng/mL folate and 20 pg/mL vitamin B12.

Performance checks for the assay include:

\# Trace binding: The CPM for the zero standard should be >35% of the CPM of the total counts. If it is <35% a failure of the microbead reagent or a procedural error may have occurred.

\# Nonspecific binding: the CPM for the blank should be <6% for the CPM of the total counts.

The accuracy of the folate assay was re-verified in 1994 with purified Sigma and Merck PGA folate standards diluted at 1.0, 5.0, 10.0 and 20.0 ng/mL dilutions. The overall slope of the regression line of the expected and calculated values was 0.985, the y-intercept was 0.3, and the r_2 was 0.9902. This procedure may be used to re-verify the kit accuracy at annual intervals. A similar procedure may be followed with purified cyancobalamin standards. Additional 160 and 320 pg/mL points are possible with the NIBSC material.

    a. Calibration of Instrument

The Packard Cobra gamma counter is used for data reduction. To ensure the accuracy of test results, take the following steps:

(1) Daily: Background and efficiency are run simultaneously using Packard Pico calibrators and associated software. Printout will indicate if all performance parameters are within acceptable limts. Efficiency should be at least 75% for I125 and 80% for Co57.

(2) Monthly: Normalization should be performed on a monthly basis. Printout will indicate if performance parameters are within acceptable limits

(3) Semi-annually: Preventative maintenance through Packard Service Inspection.

    b. Instructions for Calibration of Instrument

(1) Load Pico calibrators in positions 2,6,10,14,18 and blank tubes in positions

1,5,9,13,17 for each isotope.

(2) Insert protocol 25 clip if measuring I125, clip 26 if measuring Co57

(3) Select F2 (SC Commands), F6 (next protocol)

8. Procedure Operating Instructions; Calculations; Interpretation of Results

    a. Manual Pipetting

(1) Label 12- x 75-mm tubes in duplicate for each blank, standard, control, patient sample, and total counts. Allow all reagents and specimens to come to room temperature before use.

(2) Add 200 _L of each standard, control, or patient serum to its replicate tubes. Add 200 _L of zero standard to the blank tubes. If assaying for red cell folate, thaw the 1:11 diluted hemolysate at room temperature and mix well. Add 100 _L of hemolysate and 100 __L of red cell diluent to replicate tubes. (RBC specimens are now at the same protein levels as serum specimens in the assay.)

(3) Thoroughly resuspend the working tracer reagent and add 1.0 mL to all tubes, including the total counts tubes.

(4) Mix by vortexing each tube. Set aside total counts tubes until step (6), next section.

   b. Using Packard Multiprobe Dilutor

(1) Prepare reaction tube racks. Label 12- x 75-mm tubes in duplicate for each blank, standard, control, patient sample, and total counts tube. These tubes should be arranged in the racks according to the chosen layout. The layout information is located in the software program associated with the cesired analyte.

(2) Load samples and reagents as specified in the layout, and as required for either serum folate or the red cell folate procedure.

(3) "Execute" the protocol for the desired procedure. The parameters and values for each procedure are programmed into the protocol.

(4) Mix by vortexing the entire rack of tubes on the multi-tube vortexer. Set motor speed on "5." Using the on/off button, turn on for 2-3 sec; repeat five times for thorough mixing of the entire rack.

   c. Procedure Following Completion of Manual or Autodilutor Steps

(1) Place the tubes in the rack, cover with aluminum foil, and place the rack in a boiling-water bath at 100 _C. Allow the bath to return to a rolling boil and incubate the tubes for a minimum of 35 min. Cool to room temp by placing the rack in a cold water bath.

(2) Thoroughly mix the bottles of microbead and blank reagents by placing them on a rocker-mixer for at least 15 min prior to use. Add 100 _L of microbead reagent to each tube except the blanks. Add 100 _L blank reagent to the blank tubes. Vortex all tubes.

(3) Incubate tubes at room temperature (about 21-30 _C) for 1 hour.

(4) Centrifuge all tubes for 10 min at 1500 x g to pack the solids at the bottom of the tubes. Proceed promptly to the next step.

(5) Place the tubes in the FOAMRACS and invert the tubes over a container designated for radioactive waste in order to discard the supernatant from each tube. (A large plastic funnel or dish pan is useful for collecting the liquid and channeling it into a large plastic bottle for proper disposal of the radioactive waste.) Remove the last drops of liquid by blotting the tube rims on plastic-backed absorbent paper.

(6) Place the tubes in racks and count for 1 min in the gamma counter. Record the counts.

   d. Calculations

The Packard Cobra 10/600 gamma counter has full data reduction capabilities. Logit B/Bo vs log10 concentration is used in both counters where:

$$\text{logit } (B/Bo) = Ln((B/Bo)/(1-B/Bo))$$

and B = corrected counts/min (blank subtracted) for each tube, and Bo = maximum binding.

This method results in a linearized standard curve with an inverse relationship of levels of radioactivity (measured in counts per min, or CPM) to the concentration of folate or B12 in the serum or whole blood sample. Results are expressed as nanograms of folate per milliliter of serum (ng/mL) or as picograms of vitamin B12 per milliliter of serum (pg/mL). Red cell folate values are multiplied by 22, the dilution factor of the whole blood. The serum folate values (multiplied by 1.0 minus the hematocrit expressed as a decimal) are subtracted, and the resulting value is divided by the hematocrit to yield red cell folate in ng/mL red blood cells.

$$\text{RBC folate, ng/mL} = \frac{(\text{whole blood folate X 22}) - \text{serum folate } (1-\text{hematocrit}/100)}{\text{hematocrit}/100}$$

We recommend use of the correction for serum folate level and hematocrit because it provides the most accurate reflection of folate body stores.

   e. Special Procedure Notes – CDC Modifications

The CDC modifications for red cell folate specimen preparation necessitated by field lab collection have been included by Bio-Rad in the recommended kit instructions. The folate-only, vitamin B12-only, and folate/B12 combination kits are now used exactly as outlined by the manufacturer, usually in their entirety in one analytical run.

9. Reportable Range of Results

Values <2.0 ng/mL for serum folate, <2.0 ng/mL for whole blood folate, and <200 pg/mL for vitamin B12 are verified by re-assay. Values >20.0 ng/mL for serum or whole blood folate, or >2000 pg/mL for vitamin B12 are verified by re-assay after the solution has been diluted 1:2 with saline. These whole blood folate repeat values are approximately equivalent to 75 and 900 ng/mL RBC as red cell folate concentrations.

10. Quality Control (QC) Procedures

   a. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Inorganic Toxicology and Nutrition Branch. These specimens are prepared at two levels so as to emulate the patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

   b. Bench Quality Controls

Because of reliability and availability, three levels of Bio-Rad Lyphochek controls are

currently used as bench quality control materials. These pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The results from the pools are checked after each run. The system is declared "in control" if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met - the run is then declared "out of control":

- 🕐 1_{3s} Any of the three QC results are outside the 3s limit

- 🕐 2_{2s} Two of the three QC results in the run are outside the 2s limit (same side of mean)

- 🕐 R_{4s} Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean

- 🕐 10_x Ten sequential QC results (across pools and across runs) are on the same side of the mean

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in the analyte-specific folder on Q:/ITN/Nutrition Lab/Data handling/Import into Access. Electronic copies of the tracking of the QC results over time are stored in the analyte-specific folder on Q:/ITN/Nutrition Lab/Data handling/QC Results in Excel. A hardcopy of the QC results from each run is also kept by the analyst.

Long-term estimates of NHANES 1III assay precision in measuring serum and red cell folate during calendar year 1993 are about 3-6% coefficient of variation (CV) at 3-15 ng/mL and 5-6% at 1 ng/mL. For vitamin B12, CVs are 5-7% at 300-1500 pg/mL and 5-6% at 200 pg/mL. Representative precision and accuracy of the method are reflected in long-term quality control pool results (See Tables 2 and 3in the APPENDIX).

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria The COBRA software will allow the standard curve to be edited if there is a single outlying point. If the controls are still outside of the acceptable range, declare the system "out of control" and repeat the entire run. If the "out of control" condition still exists, use a new kit and evaluate the pipetting precision and accuracy of the autodiluter.

Reassay specimens for that analytical run after the system has been verified to be "in control," and report the results of the reassay rather than the original values.

If the steps outlined above do not result in the correction of the "out of control" values for QC materials, consult the supervisor for other corrective actions. The ICN analyzer may be used if the Packard is non-functional.

12. Limitations of Method; Interfering Substances and Conditions

The most common causes of imprecision are intermittently inaccurate micropipettors, inadequate boiling time, and microbead reagent that was not mixed thoroughly before it was added to the solution.

Hemolyzed serum samples may give falsely elevated values. Exposure to strong sunlight for more than eight hours may cause 10-20% serum folate degradation.

13. Reference Ranges (Normal Values)

Current proposed normal ranges for serum folate are about 2.6-12.2 ng/mL for both sexes and all ages. Values are lower in females than in males. Serum levels of 1.4-2.6 ng/mL are usually termed "indeterminate" because of an overlap between "deficient" and "normal" ranges. Serum folate values <1.4 ng/mL are usually indicative of inadequate folate intake. Elevated values are caused by supplementation. (See NOTE below.)

Red cell folate values are more indicative of body stores, whereas serum levels reflect only recent dietary intake. The approximate normal range for red cell folate is 102.6-410.9 ng/mL RBC.

The expected normal range for vitamin B12 is about 165-1600 pg/mL. Concentrations of 160-200 pg/mL are considered "indeterminate", and those less than 160 pg/mL are considered deficient. Elevated B12 levels may result from over supplementation or may reflect myeloproliferative disorders.

Values from NHANES 1999+ will be used to update the U.S. national normal ranges. Results from NHANES III are published in the Series 11 Report.

Extensive evaluation by CDC, Cambridge University, and Stanford University researchers, showed PGA calibrators in original Quantaphase kits to be inaccurate by an average of 32.5% across 0-20 ng/mL range. Using freshly received NHANES 1999+ specimens, CDC performed 19 comparison runs with "old" and "new" calibrators (with spectrophotometrically verified concentrations of PGA) to establish a correction factor. The equation used for the correction was:

Log_{10} (corrected value) = -0.1956 + 1.0199 Log_{10} (uncorrected value)

Or, expressed in linear terms:

corrected value = -0.1411 + 0.6849 x (uncorrected value)

Evaluations were also made with N_5MeTHFA standards. No change occurred to affect vitamin B12 results.

The currently used reference ranges for serum and red cell folate are based on a mathematical correction of previously recommended values from the supporting documentation for HANES II and reflect the approximately 32.5% average difference for standards between current versions of the Quantaphase II folate kits. All of Phase I of NHANES 1999+ was performed with the original kit; hence, data from Phase I were corrected to correspond with the data from Phase II.

14. Critical Call Results ("Panic Values") Any NHANES samples with serum folate levels <1.5 ng/mL, RBC folate levels <75 ng/mL RBC, or B12 levels <200 pg/mL are considered to require follow-up. Since survey data are transmitted several times weekly to Westat, abnormal reports are automatically forwarded to the NCHS survey physician for follow-up. For smaller, non-NHANES studies, abnormal values are identified to the study principal investigator. Most of these studies are epidemiological in nature, however.

15. Specimen Storage and Handling During Testing

Specimens should be allowed to warm to and be maintained at room temperature during preparation and testing.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

There are no acceptable alternative methods of analysis for folate or vitamin B12 in the NHANES laboratory. The ICN may serve as an alternative gamma counter; no substitution is permitted for the Quantaphase II assay.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX by the supervisor of any serum folate result that is <1.5 ng/ml,

RBC folate that is <75ng/ml and B12 <200 pg/ml, possibly represents a significant risk for B vitamin deficiency. Copies of FAXes sent concerning abnormal results are kept in a notebook by the supervisor for the duration of the study.

Test results that are not abnormal are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically several times weekly to the Westat ISIS computer and then are transferred to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

The Microsoft Access database is used to keep records and track specimens for

NHANES 1999+. If plasma or serum folate/B-12 analyses are used for smaller,

non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab on the DLS LAN.

We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -70 _C. The specimen ID is read off of the vial by a barcode reader attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

Helicobacter Pylori IgG Antibodies in Serum by Enzyme Immunoassay

Helicobacter pylori in Serum NHANES 1999-2000 3 1. Summary Of Test Principle And Clinical Relevance
The Wampole Laboratories (Wampole) H. pylori IgG Enzyme-Linked Immunosorbent Assays (ELISA) is intended for the detection and qualitative determination of IgG antibodies to *Helicobacter pylori* in human serum. This assay is intended for use as an aid in the diagnosis of *H. pylori* infection in persons with gastrointestinal symptoms. For *in vitro* diagnostic use only.

Enzyme linked immunosorbent assays (ELISA) rely on the ability of biological materials, (i.e. antigens) to adsorb to plastic surfaces such as polystyrene (solid phase). When antigens bound to the solid phase are brought into contact with a patient's serum, antigen-specific antibody, if present, will bind to the antigen on the solid phase forming antigen-antibody complexes. Excess antibody is removed by washing. This is followed by the addition of goat anti-human IgG conjugated with horseradish peroxidase which then binds to the antibody-antigen complexes. The excess conjugate is removed by washing, followed by the addition of chromogen substrate tetramethylbenzidine (TMB). If specific antibody to the antigen is present in the patient's serum, a blue color develops. When the enzymatic reaction is stopped with 1N H2SO4, the contents of the wells turn yellow. The color, which is proportional to the concentration of antibody in the serum, can be read on a suitable spectrophotometer or ELISA microwell plate reader. The sensitivity, specificity, and reproducibility of enzyme-linked immunoassays can be comparable to other serological tests for antibody, such as immunofluorescence, complement fixation, hemagglutination and radioimmunoassays.

Helicobacter pylorus (previously Campylobacter pylori) is a spiral bacterium that was cultured from the human gastric mucosa in 1982. Various studies have indicated that the presence of *H. pylori* is strongly associated with chronic (Type B) gastritis. *H. pylori* colonization is usually chronic in nature. If the organisms are eradicated, the

histological inflammation improves. When the organisms reappear inflammatory changes recur. These findings have favored the theory that chronic colonization by *H. pylori* causes Type B gastritis. Even though there is histological inflammation, symptoms are frequently not present. The presence of *H. pylori* has also been associated with gastric and duodenal ulcers. The organism is present in 95-98% of patients with duodenal ulcers and 60-90% of patients with gastric ulcers. A person with gastrointestinal symptoms with evidence of *H. pylori* colonization (i.e. presence of specific antibodies, positive breath test, positive culture or positive biopsy) is considered to be infected with *H. pylori*. A person without gastrointestinal symptoms having evidence of the presence of the *H. pylori* organism is said to be colonized not infected. Studies have demonstrated that removal of the organism by antimicrobial therapy reduces the risk of peptic ulcer recurrence and relieve symptoms.

Traditionally, the presence of *H. pylori* has been detected through biopsy. The biopsy is obtained by endoscopy. As with any invasive procedure needing some form of sedation, some risk and discomfort to the patient is present. Detection of the organism involves culture of the gastric biopsy specimen, examination of stained biopsies for the presence of bacteria, or detection of urease activity in the biopsies themselves. Biopsy by endoscopy may lack some sensitivity due to the patchy nature of *H. pylori* colonization. Noninvasive methods include a urea breath test, which utilizes isotopes allowing for the detection of urease activity produced by the organism, and serology. The presence of *H. pylori* specific IgG antibodies in human serum has been shown to be associated with past or present *H. pylori* colonization.

2. Safety Precautions

Consider all samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Wear gloves, lab coat, and safety glasses when handling all human blood products and infectious viruses. Place disposable plastic, glass, paper, and gloves that contact blood in a biohazard bag or discard pan to be autoclaved. Disinfect all work surfaces with a 1:200 dilution of Staphene (Calgon Vestal Laboratories, St. Louis, Missouri). Dispose diluted specimens and any other potentially contaminated materials in a biohazard bag at the end of the analysis to be autoclaved prior to final disposal. Autoclaved or disinfect other non-disposable material at the end of the working day.

Do not pipette by mouth. Do not eat, drink or smoke in designated work areas. Wash hands thoroughly after removal of personal protective devices used in handling specimens and kit reagents.
Material safety data sheets for all reagents used in the performance of this assay, including but not limited to Staphene, sodium hydroxide, sodium hypochlorite, and sodium azide, are kept in the Immunology Division, University of Washington Medical Center (UWMC).

3. Computerization; Data System Management

a. Each shipment of specimens received from the NHANES IV mobile unit arrives with a corresponding transmittal sheet and a Send File (a comma delineated text file) transmitted electronically (labeled *boxnum.*shp). This file contains the following information:
Send File

Field	Type
Sample ID	XXXXXXX
Slot Number	XXX
Sample Collection Date	Mm/dd/yyy hh:mm:ss
MEC Comment Code	XX

b. The information from the shipping file is imported into a result file with the following format:

Results File: CRP/H. Pylori-Vessel ID 13

Field	Format	Type	Item ID
Sample ID	XXXXXXXX	Int	
Slot Number	XXX	Smalint	
Sample Collection Date	mm/dd/yy hh:mm:ss	Smalldatetime	
MEC Comment Code	XX	Smalint	
Date of Receipt	mmddyy	Smalldatetime	LBXCRPDR
BN2 CRP Run num	{test code} mmddyy. {a,b,c}	Char (10)	LBXCRPBT
Date of BN2 CRP Analysis	mmddyyy	Smalldatetime	LBXCRPDA
BN2 CRP	XXXX.XX	Char (8)	LBXCRP
BN2 CRP Comment	XX	Smallint	LBXCRPLC

Field	Format	Type	Item ID
BN2 CRP analyst id	XXX	Char (3)	LBXCRPTK
BN2 CRP 2.5% repeat	XXXX.XX	Char (8)	LBCCRP
Wampole H.pylori run_num	{test code} mmddyy. {a,b,c..}	Char (10)	LBXHPBT
Date of Wampole H.pylori analysis	mmddyyyy	Smalldatetime	LBXHP1DA
Wampole H. pylori	XXX.XX	Numeric(5,2)	LBXHP1
Wampole H.pylori Comment	XX	Smallint	LBXHP1LC
Wampole H. pylori analyst ID	XXX	Char (3)	LBXHP1TK
Wampole H. pylori 2.5% repeat	XXX.XX	Numeric(5,2)	LBCHP1

c. After the testing is completed, the run number, date of analysis, H.pylori result, H. pylori comment, H.pylori analyst, and the H.pylori 2.5% repeat results are entered into the result file.

d. Data entry is checked for errors.

e. After the C reactive protein testing has also been completed, resulted, and checked, the result file is transmitted electronically to NHANES WESTAT. Electronic and hard copies of the files are kept in the laboratory.

f. Technical support for this system is provided by Westat, Rockville, MD (1-301-294-2036)

4. Specimen Collection, Storage, And Handling Procedures; Criteria For Specimen Rejection

   a. No special instructions such as fasting or special diets are required.
   b. Fresh or frozen human serum, heparin and EDTA plasma samples are acceptable. Specimens should be frozen at $\leq$-20 ^{o}C if testing is not done within 24 hours of collection.

    c.   Blood should be collected aseptically and the serum separated by standard laboratory techniques. Specimens may be collected by using regular or serum-separator Vacutainers. Serum or plasma should be separated from the cells within 60 minutes of collection.

    d.   The requested sample volume for the assay is 1.0 mL, and the minimum sample volume is 0.3 mL.

    e.   Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

    f.   Contamination or introduced particulate matter can lead to erroneous results. Heat inactivated specimens should not be used. Very lipemic specimens should be clarified by centrifugation (10 minutes at approximately 15,000 g) prior to testing

    g.   Avoid repeated freeze/thaw cycles.

5. Procedures For Microscopic Examinations; Criteria For Rejection Of Inadequately Prepared Slides
Not applicable for this procedure.

6. Preparation Of Reagents, Calibrators (Standards), Controls, And All Other Materials; Equipment And Instrumentation

    a.   Reagents and Standard Materials.

    1.   Wampole H. pylori kit# 446401, (Wampole Laboratories, Division of Carter Wallace, Inc., Cranbury, NJ) Store all unopened kit components between 2o and 8oC. The test kit may be used throughout the expiration date of the kit. Refer to the package label for the expiration date. Return all opened kit components as quickly as possible to storage between 2o and 8oC. Contents are as follows:

    H. *pylori* (strain ATCC 43504) antigen coated microassay plate: 96 wells, configured in twelve 1x8 strips. (96T: one plate) Unused strips must be immediately resealed in a sealable bag with desiccant/humidity indicator, and returned to storage at 2o and 8oC.

    Serum Diluent: Ready for use. Contains Proclin (0.1%)as a preservative, pH 7.5$\pm$0.2. (96T: one bottle, 30 mL)

    Calibrator: human serum. Sodium azide (0.1%) and pen/strep (0.01%) added as preservatives, with kit specific factor printed on vial label. The Calibrator is used to calibrate the assay to account for day-to-day fluctuations in temperature. (96T: one vial, 0.25 mL)

    Horseradish-peroxidase (HRP) Conjugate: ready to use. Goat anti-human IgG, containing proclin (0.1%) as a preservative. (96T: one bottle, 16 mL)

    Chromogen/Substrate Solution: Tetramethylbenzidine (TMB), ready to use. (96T: one bottle, 15 mL)

    Wash Buffer (20X concentrate): dilute 1 part concentrate + 19 parts deionized or distilled water. Contains TBS, Tween and Proclin (0.1%) as a preservative, pH 7.2 $\pm$ 0.2. (96T: one bottle, 60 mL) Store 1X (diluted) wash buffer at room temperature (21° to 25° C) for up to 5 days, or 1 week between 2o and 8oC.

    Stop Solution: Contains an H_2SO_4 solution, ready to use. (96T: One bottle, 15 mL)

2. Deionized water (University of Washington Medical Center, Seattle, WA)

    b.   Reagent Preparation

1. Allow all reagents to equilibrate to room temperature (18 – 28 º C) for at least one hour before performing the assay.

2. Prepare the working strength wash solution. Dilute the 20x wash concentrate with deionized water (1 part 20x wash concentrate to 19 part deionized water). Mix for a minimum of 5 minutes. Stable for 5 days at room temperature or 1 week between 2º and 8º C.

    c.   Instrumentation

1. Bio Tek EL 808 Automated Microtiter Plate Reader - is a 8 channel, automated, benchtop, general purpose, enzyme immunoassay analyzer which measures the optical density of solutions in a 96-well microtiter plate at 405nm, 414nm, 450nm, 550nm, or 650nm. Data handling and reduction is performed using Bio Tek KC4 KinetiCalc for Windows. This software is a general data reduction package used to analyze data generated from colorimetric microtiter plate assays as read on the Bio Tek EL808. (Bio Tek Instruments, Winooski, Vermont) This instrument includes a Hewlett Packard Laser Jet 6MP printer (Hewlett Packard, Boise ID).
OR
1. Rosys Plato 3301 is fully automated, benchtop, general purpose, enzyme immunoassay analyzer which performs all sample, dilution, plate rotation, and reagent handling steps including movement of plates, mixing of samples, incubation, washing and measuring the optical density of solutions in a 96-well microtiter plate at 405nm, 414nm, 450nm, 550nm, or 650nm. Data handling and reduction is performed using resident Rosys Plato 3301 software (Rosys Anthos Instruments, New Castle, DE) This instrument includes a Hewlett Packard Laser Jet 6MP printer (Hewlett Packard, Boise ID).

2. Air Driven Ultracentrifuge, model 340400 (Beckman Instruments, Fullerton, CA).

3. Computer (Dell Computer Systems, Round Rock, TX).

4. Digiflex Automatic Pipettor (#33010, ICN Micromedic Systems, Inc., Huntsville, AL) – is a precision liquid delivery instrument which is used as a sample dilutor.

5. Disposable tip precision pipettors: fixed volume or adjustable for 50, 100, and 150 uL ($\pm$1%) Optionally, a multichannel pipette can be used together with disposable V-shaped troughs for addition of goat anti-human IgG assay conjugate, substrate and quench reagents.

6. Repeating pipettors and disposable tips for 50, 100 and 300 uL (Oxford Labware, San Francisco, CA).

7. Skatron Macrowell Tube Strips (1.0 mL minitubes), cat# 5776 (Genetic Systems Sanofi, Woodinville, WA)

8. Nunc Immuno Wash- a microplate washer and aspiration device (Nunc Immuno Wash #470173, through VWR #62409-148)

9. Timer (any vendor)

10. Container for preparation of wash solution, 500 ml, (any vendor)

11. Reagent boats for containing conjugate, substrate and stop solutions, (any vendor)

12. Plate Sealers, cat# 3095 (Corning Inc., Corning, NY)

    d.   Standards/Calibrator Preparation

Assay calibrators are received in a liquid ready to use format. No further preparation is required prior to use other than bringing to room temperature (18 ^{o}C -28 ^{o}C).

e. Preparation of Quality Control Materials

The Immunology Division prepares two levels of control from normal and/or pooled patient sera. Both pools are analyzed with each assay.

Prepare in sufficient quantity to provide control material for at least 2 years. Prior to aliquoting and defining, test the stock once for approximate value and adjust if necessary.

Analyze newly prepared control material for at least 20 runs in parallel with the current control to determine acceptance ranges. Acceptance ranges must be determined prior to using control material for any patient run evaluations.

Divide the stock control material into 10-mL tubes containing a volume for a 3-4 month supply and label with 'I #' and freeze at $\leq$ -70 ^{o}C. As needed, thaw a stock control tube and divide into approximately 100 uL aliquots to be stored for a maximum of 3-4 months at -70 ^{o}C. Thaw and use one aliquot of control material for each run.

1. As new stock control is prepared, define a new control range by assigning the first value observed as the mean and assigning a large standard deviation. Append TEMP to the control lot number name. Prepare new blank Levey-Jennings table using these temporary limits.
2. After 20 parallel runs, use the data from the Levey-Jennings chart to assign a permanent mean and standard deviation. Normal acceptance ranges are determined as mean $\pm$ 2 standard deviations.
3. Stock control material is aliquoted into individual use bullets. The aliquot bullet label should include the date of preparation and a letter indicating sequential aliquot. (Examples: 9/90-A for the first time this control is aliquoted, 9/90-B for the second time. Record the label on the quality control material record sheet.
4. The lot name should include an identifying name, the date the control was prepared (month and year), and information about the control range (temp or date of calculation or recalculation).

7. Calibration And Calibration Verification Procedures
    1. Calibrator
    A cutoff calibrator, with kit specific factor printed on vial label, is run in triplicate with each run. Each cutoff calibrator must be >/=0.250A at 450 nm (when read against the reagent blank). The mean value for the run is calculated. If any of the three Cutoff calibrators' values differ by more than 15% from the mean, that value is discarded and the mean of the two remaining values is calculated.

    a. Verification

    The instruments used to read assay results are equipped to analyze the two different level controls for each test series. If, within a testing series, these controls do not conform to specifications as defined in the quality control manual, the entire series is invalidated.

    8. Procedure Operating Instructions; Calculations; Interpretation Of Results

    a.   Preliminaries

        1. The procedure for the Wampole H.pylori assay is performed at room temperature. Bring all kit components to room temperature (18 ^{o}C -28 ^{o}C) and gently mix before use.

2. Bring serum specimens to room temperature and mix well.

3. Dilute test sera, Cutoff Calibrator, and Control sera 1:21 (e.g., 10 uL + 200 uL) in Serum Diluent.

4. Remove the appropriate number of microwell strips from the sealed pouch. Allow six control/cutoff calibrator determinations (a reagent blank, a negative control, a positive control, and a cutoff calibrator run in triplicate. Patients are run in singlicate). Promptly return unused strips to pouch with desiccant.

5. Prepare adequate wash solution for the run (dilute 1 part concentrate + 19 parts deionized water).

6. All calibrators, controls, and specimens should be tested at the same time and run in duplicate. Because the termination of each incubation stops a reaction that is in progress (i.e., antibody binding or substrate turnover), reliable calibration of the assay depends on ensuring that the incubation times are essentially the same for all wells.

b. Assay procedure:

1. Pipette 100 uL diluted Cutoff Calibrator, Controls and specimens to antigen coated microwells, using a multichannel pipette. Add 100 uL of Serum Diluent to the reagent blank well.

2. Incubate each well at room temperature (21 ^{o}C -25 ^{o}C) for 20 minutes +/- 2 minutes. Apply a plate sealer and swirl the plate gently on a flat surface for 5-10 seconds to mixing.

3. At the end of the incubation period, carefully remove and discard the plate sealer. Using a microtiter plate washing device, aspirate the liquid then dispense Wash Solution into the first strip, move to the next strip and do the same. Complete all strips in the plate in this manner. Return to the first strip and repeat this procedure on the entire plate 2 more times. After completing the third wash cycle, aspirate all liquid out of each strip, then pound out any excess liquid onto a stack of paper towels. Immediately proceed to the next step, do not allow the plate to dry.

4. Add 100 uL Conjugate to each well, including reagent blank well. Avoid bubbles upon addition as they may yield erroneous results. Cover the plate with a plate sealer.

5. Repeat wash step as described in step 3.

6. Add 100 uL chromogen substrate solution to each well, including reagent blank well, maintaining a constant rate of addition across the plate.

7. Incubate each well at room temperature (21 ^{o}C -25 ^{o}C) for 10 minutes +/- 2 minutes.

8. Stop the reaction by addition of 100 uL of Stop Solution following the same order of chromogen substrate addition including the reagent blank. Tap the plate gently along the outsides to mix contents of the well.

9. Wait a minimum of 5 minutes and the read the absorbance of each well at 450 nm. The plate may be held up to 1 hour after addition of the Stop Solution before reading.

c. Calculations:

1. Mean Calibrator O.D. - Calculate the mean value for the Calibrator from the three Calibrator determinations. If any of the three Calibrator Values differ by more than 15% from the mean, discard that value and calculate the mean of the two remaining values.

2. Correction Factor - To account for day-to-day fluctuations in assay activity due to room temperature and timing, a Correction Factor is determined for each lot of kits. The Correction Factor is printed on the Calibrator vial.

3. Cutoff Calibrator Value - The Cutoff Calibrator Value for each assay is determined by multiplying the Correction Factor by the mean Calibrator O.D. determined in step 1.

4. Immune Status Ratio - Calculate an Immune Status Ratio (ISR) for each specimen by dividing the specimen O.D. value by the Cutoff Calibrator Value determined in step 3.

Example:
O.D.s obtained for Calibrator = 0.38,0.40,0.42
Mean O.D. for Calibrator = 0.40
O.D. obtained for patient serum = 0.60
Correction Factor = 0.50
Cutoff Calibrator Value = 0.50 x 0.40 = 0.20
Immune Status Ratio = 0.60/0.20 = 3.0

d. Recording of Data

1. Analytical Results Data

Specimen results are entered into the assay specific results table created from the send file corresponding to the specific sample box using Excel software (Microsoft Corporation, Redmond WA). A copy of this table is printed out and checked for accuracy of data entry.

2. Quality Control Data

Control results are entered into the assay specific Levey Jennings table and plot if they are found to be in compliance with Westgard rules The evaluated copy of the table is printed out and checked for accuracy of data entry.

9. Reportable Range Of Results

Report values to the nearest 0.01. The lowest reportable range is from 0.0. The upper reportable value is determined by the absorbance reading capacity of the plate reader and will vary from day to day. Absorbances exceeding this upper reading capacity are repeated on dilution on a following run until observed absorbance value is within the readable range of the plate reader.

10. Quality Control (Qc) Procedures

a. Good laboratory practices include the use of control specimens within an assay run to ensure that all reagents and protocols are performing properly.

b. Recovery of control concentration should fall within the stated range. If the controls are out of range:

- Verify that the microplate reader is correctly programmed.

- Verify that the controls have not exceeded the expiration date.

- Check the control ranges for accuracy.

- Verify that the order of addition has not been changed.

c. A cutoff calibrator, with kit specific factor printed on vial label, is run in triplicate with each run. Each cutoff calibrator must be >/=0.250A at 450 nm (when read against the reagent blank). The mean value for the run is calculated. If any of the three Cutoff calibrators' values differ by more than 15% from the mean, that value is discarded and the mean of the two remaining values is calculated.

d. All pipettes used in testing clinical specimens should be checked for calibration every 3 months.

e. Recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipette by serial number.

f. Control results are evaluated by Westgard rules for each run by entry into Levey Jennings plot tables. Levey Jennings graphs are evaluated prior to reporting of any patient samples. Any violations of control specifications should be referred to supervisor.

g. Estimates of imprecision can be generated from long-term quality control pool results. Bench quality controls are used in this analytical method. Bench quality control specimens are inserted by the analyst at least once in each analytical run (a set of consecutive assays performed without interruption) so that judgments may be made on the day of analysis. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

h. The bench controls are prepared in sufficient quantity to provide samples for all the assays for 2 years. Ranges are established after 20 parallel runs with previously established controls. Ranges are established by using the formulas for statistical calculation data. The quality control pools comprise two levels of concentration spanning the low and high ranges for antibodies to H. pylori.

i. Calibrator and bench quality controls are placed at the beginning of each analytical run. After analysis, the long-term quality control charts (Levey-Jennings) for each control material are consulted to determine if the system is "in control." The Levey Jennings chart plots the quality control material observation on the y-axis and the date of the observation on the x-axis. Quality control material observations are compared with the 95% and 99% confidence limits as well as with the center line (the overall mean of the characterization runs) prior to reporting any results. The system is out of control if any of the following events occur for any one of the quality control materials:

- The observation from a single pool falls outside the 99% confidence limits.

- The observations from two pools fall either both above or both below the 95% confidence limits.

- The observations from eight successive runs for one pool fall either all above or all below the center-line.

11. Remedial Action If Calibration Or Qc Systems Fail To Meet Acceptable Criteria

If the run is declared "out of control", the system (instrument, calibration standards, reagents etc.) are investigated to determine the root of the problem before any results are released. Consult with the supervisor for appropriate actions.

12. Limitations Of Method; Interfering Substances And Conditions

a. The user of this kit is advised to carefully read and understand the package insert. Strict adherence to the protocol is necessary to obtain reliable test results. In particular, correct sample and reagent pipetting, along with careful washing and timing of the incubation steps are essential for accurate results.

b. Icteric, lipemic, hemolyzed or heat inactivated sera may cause erroneous results and should be avoided if at all possible.

c. The performance characteristics have not been established for any matrices other than sera.

d. The values obtained from this assay are intended to be an aid to diagnosis only. Each physician must interpret the results in light of the patient's history, physical findings and other diagnostic procedures.

e. The assay should be performed only on patients with gastrointestinal symptoms due to the large percentage of *H. pylori* colonized individuals, especially in the older age groups.

f. Positive result indicates that the patient has antibody to *H. pylori*. It does not indicate that any existing symptoms are due to *H. pylori* infection or colonization. It also does not differentiate between active or past infection. The clinical diagnosis has to be interpreted with clinical signs and symptoms of the patient.

g. A negative result indicates that the patient does not have detectable levels of antibodies to *H. pylori*. If a sample is drawn too early in *H. pylori* colonization, IgG antibodies may not be present. The clinical diagnosis has to be interpreted with the clinical signs and symptoms of the patient.

h. Performance characteristics have not been established with patients under the age of 18.

i. The Calibrator and Controls contain sodium azide, which may inhibit the conjugate activity if the washing steps are not properly performed.

13. **Reference Ranges (Normal Values)**

a. The patients ISR (Immune Status Ratio) are interpreted as follows:

ISR	Results	Interpretation
$\leq$0.90	Negative	No detectable IgG antibody by the Elisa test.

0.91-1.09	Equivocal	Samples that remain equivocal after repeat testing, should be repeat testing, should be restricted by an alternate method, e.g., alternate ELISA assay. If the results remain equivolcal upon further testing, an additional sample should be taken.
>1.10	Positive	Indicates Presence of IgG

       b.   To determine the cut-off of the assay, 101 negative sera were assayed by the Wampole H. pylori IgG ELISA test. The negativity and positivity of specimens used to determine the cut-off for the assay were determined by biopsy. The mean and standard deviation of the optical density readings for the sera was 0.131 and 0.109 respectively. The positive threshold for the assay was determined by adding the mean and three standard deviations (0.131 + 3 (0.109) = 0.46). A positive serum was titrated to give a constant ratio of the threshold value to obtain a cut-off Calibrator serum. On all subsequent assays this serum was run and the assay was calibrated by multiplying the O. D. value for the cut-off Calibrator by the ratio to the cut-off to obtain the cut-off O.D. This value was then divided into the O.D. for the patient sera to obtain an Immune Status Ratio (ISR). By definition the cut-off ISR is equal to 1.00. To account for inherent variation in immunoassay, values of 0.91-1.09 were considered equivocal. Therefore values <0.90 are considered negative and the values >1.10 are considered positive.

       c.   Virtually all H. pylori infected persons possess IgG antibodies to H. pylori. The prevalence of H. pylori infection found in individuals with related clinical conditions is found in Table 1 below. Because the presence of the H. pylori organism is so common and because the simple colonization is asymptomatic (vs symptomatic infection), many individuals apparently free of gastrointestinal systems are antibody-positive. The height of antibody response is not correlated with the presence or severity of symptoms. The prevalence of H. pylori antibodies rises with age as shown in Table 2. H. pylori antibodies are found in men and women at equal rates; Blacks, Hispanics, and persons born outside the United States show higher rates of colonization.

Table 1 - Prevalence of H. pylori in Persons with Related Clinical Conditions

Diagnosis	Incidence
Chronic active (Type B) gastritis	95-100%
Duodenal Ulceration	96-98%
Gastric Ulceration	60-90%
Non-ulcer dyspepsia	50-75%
Pernicious anemia (Type A) gastritis	0-20%

Table 2 - Prevalence of H. pylori Antibodies Found in Asymptomatic Caucasians in the U.S.

Age (years)	Prevalence
0-19	<10%
20-29	5-20%
30-39	10-30%
40-49	20-40%
50-59	30-50%

60+	40-60%

14. **CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable to this procedure.

15. Specimen Storage And Handling During Testing

Specimens should be maintained at 20-28 ^{o}C during testing. After testing, the samples are stored at $\leq$ -70 ^{o}C.

16. Alternative Methods For Performing Test Or Storing Specimens If Test System Fails

There are no acceptable alternative methods of analysis. Specimens may be stored at 4-8 ^{o}C for no longer than 72 hours. Otherwise, specimens should be stored at $\leq$ -70 ^{o}C until the system is returned to functionality.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)
Not applicable to this procedure.

18. Transfer Or Referral Of Specimens; Procedures For Specimen Accountability And Tracking

Standard record keeping should be used for tracking specimens. The primary results include daily test results as well as stored quality control results.

The original NHANES IV ship file is copied into a template Excel file and onto the hard drive of a PC computer. After the results are entered into the database and assay results transmitted electronically. Files are stored for 6 months on a server that is backed up on a daily basis. After 6 months, the result files are transferred onto a CD along copies of original ship files and QC information.

The residual serum is stored at $\leq$ -70 ^{o}C for 6 months after analysis, then it is returned to the NHANES Repository in Rockville, MD for long-term storage.

Panic Disorder Question-by-Question Objectives (Section of CIDI)

A. Introduction

The essential feature of Panic Disorder is a panic (anxiety) attack that occurs suddenly and unpredictably, though certain situations, e.g., driving a car, may become associated with a panic attack. The same symptoms occurring during marked physical exertion or in a life threatening situation is not termed a panic attack.

A panic attack is manifested by a sudden onset of intense apprehension, fear, or terror, often associated with feelings of impending doom. The most common symptoms experienced during an attack are: shortness of breath; heart pounding; chest pain or discomfort; choking or smothering sensations; nausea or abdominal distress; dizziness; feelings of unreality; tingling in the hands or feet; hot flushes or chills; sweating; faintness; trembling or shaking; dry mouth; and fear of dying, going crazy, or doing something uncontrollable during the attack. Attacks usually last minutes, rarely hours.

Panic attacks are classified into those which occur only in the presence of the object of a phobia and those which occur at other times.

105

If the respondent indicates that (he/she) has had only one attack in the past 12 months and it was due to a life-threatening situation, the respondent meets inclusion criteria and is taken through most of this section. The respondent then may (depending on the response pattern) be taken through the work loss and cutback day questions which will ask about the one incident. This may seem redundant to the respondent, but the interviewer should tell the respondent that each question needs to be asked in the order in which it appears in the questionnaire.

B. Question-by-Question Specifications

D54   The interviewer should emphasize the words, <u>sudden</u> and <u>felt frightened, anxious or very uneasy</u>.

D54.1   This is a "second chance" question, which asks about panic attacks in a slightly different way. The purpose of this question is to make certain that no one is skipped out of this section in error.
The phrase "out of the blue" means "for no apparent reason"

D54b   This question refers to the respondent's <u>concern</u> about having another attack, whether or not an attack actually occurred.

D54c.1 The "ongoing concern" in this question refers to the concern mentioned in D54c, "that the attacks might lead to something terrible happening."

D55   This question ascertains whether any of the panic attacks someone has had occurred in a life-threatening situation. The definition of "life-threatening" is whatever the respondent thought was life threatening at the time of the incident.

D57   This question refers only to symptoms that occurred during the most recent panic attack that was not in a life-threatening situation.
Card A is handed to the respondent at the beginning of this question. The interviewer should read each item to the respondent, even if the respondent volunteers that (he/she) has had some of the symptoms on the card. For items that have more than one option (e.g., ""hot flushes" or "chills"), a "yes" to either option counts as a "yes" for that item.

D59   Note that this question refers to the number of panic attacks the respondent has had in his/her entire lifetime. To qualify, the attacks must be accompanied by some of the symptoms listed in D57.
This can be a difficult question for respondents, especially if they have had many panic attacks over a long period of time. If necessary, probe "What's your best estimate?" to encourage the respondent to come up with a number. Code "900" for 900 or more attacks. In the follow-up questions that refer to these attacks, the text will show up as "900" on the computer screen, and the interviewer can modify the question to read "900 or more" where appropriate.

D59.2c This question is very lengthy and complex. Read slowly, and repeat the key phrases and/or read the last phrase in parentheses if the respondent has not understood the entire meaning of the question.

D59.2d If the respondent has had a panic attack in a situation where the respondent had an unusually strong fear, this question asks what that fear is. Probe to determine in which situation the panic attack occurred if not volunteered by respondent.
If the respondent mentions a fear of something not on the list, code "other" and record the fear in the text box.

D60   Emphasize the words <u>exact</u> and <u>very first</u> when reading this question.

D60c   This question is very similar to D60. There is a subtle difference (D60 asks the respondent to recall (his/her) <u>exact age</u> at (his/her) first panic attack; and D60C asks the respondent to recall the earliest age (he/she) can <u>clearly remember</u> an attack). It is possible that the respondent knows (he/she) had panic attacks at a very young age, but does not remember them clearly.

D59a   This is a complicated question. Read slowly. The respondent is asked to divide the total number of panic attacks in (his/her) lifetime into those that occur "out of the blue" (for no particular reason), in situations of unreasonably strong fear, and in situations of real danger.
There is no check to be sure that the sum of the panic attacks in these three situations adds up to the total number of panic attacks reported by the respondent. If the respondent has obviously misunderstood the question, probe to get an accurate number; but do not attempt to force the numbers to add up to the total number of attacks.

D59d   If the respondent has had panic attacks in situations where the respondent had an unusually strong fear, this question asks what those fears are. Probe to determine in which situation(s) the panic attacks occurred if not volunteered by respondent. Probe "any others?" until the respondent answers "no," and check all that apply.

               If the respondent mentions a fear of something not on the list, code "other" and record the fear in the text box.

D59.5d   Code "900" for 900 or more attacks.

D59.5e   A "four-week period" in this question refers to four consecutive weeks. Code "900" for 900 or more attacks.

D59.5f   Read this question slowly. To code "yes," the respondent must have had four or more attacks every week for four consecutive weeks.

D58a   This question begins the Probe Flow series of questions.

D62   This is a long and complicated question. Read slowly. The question asks the respondent to indicate how much the panic attacks have interfered with (his/her) life in any way. If the respondent replies that some attacks interfere a lot and others do not, probe "In general, how much did these things interfere with your life or activities in the past 12 months?"

               "Incapacitating" is defined as "making someone unable to do anything."

D62a   The "problems" that this question refers to are those listed in the previous question:

            • the incapacitating effect of the attacks themselves,

            • worry about the attacks getting in the way of daily activities, or

            • avoiding certain situations (for fear of having additional attacks) that interferes with daily activities.

The respondent must be totally incapacitated for an entire day to have the day counted for this question. If the respondent reports being totally incapacitated only for a few minutes (or hours) during and after the panic attack, this day would be counted in D62b (the "cut back" question).

Generalized Anxiety Disorder (Section of CIDI)

A. Introduction

The essential feature of this disorder is unrealistic or excessive anxiety and worry about two or more life circumstances for six months or more, during which the person has these concerns more days than not. Accompanying the anxiety are many signs of motor tension, autonomic hyperactivity, and vigilance and scanning.

B. Question-by-Question Specifications

D63   It is important to read this question slowly and carefully.

D64d   This question asks the respondent to indicate the types of things that (he/she) worried about. The interviewer is instructed to probe "Anything else?" until the respondent answers "No."

D64d.1   This item is the first of a series of questions to be coded by the interviewer based on the respondent's answer to D64d. The answers to these checkpoints will determine the skip patterns for the remainder of the section. These questions are not read aloud to the respondent, and the interviewer may use task-related feedback such as "It will be just a moment while I code a few items on the computer," to fill the gap in the questioning.

D65.1   Hand Card B to the respondent prior to asking this question. Each item should be read aloud to the respondent even if (he/she) volunteers an answer from the card before the item is read.

D66   This is the start of the Probe Flow questions.

D69c   This question is very similar to D69. There is a subtle difference (D69 asks the respondent to recall (his/her) exact age at (his/her) first period of worry, tension, or anxiety; and D69C asks the respondent to

recall the earliest age (he/she) can <u>clearly remember</u> such a period). It is possible that the respondent knows (he/she) had periods of worry, tension or anxiety at a very young age, but does not remember them clearly.

<u>Major Depression (Section of CIDI)</u>

A. Introduction

A Depressive Episode consists of at least two weeks characterized by feelings of sadness or lack of interest or pleasure, along with other symptoms such as poor appetite, difficulty sleeping, feelings of worthlessness or guilt, decreased energy, and thoughts of death.

Synonyms for Depression

There are three different questions that are used to measure depression, because people use many different terms to describe the condition. E1 asks about low mood and other synonyms for depression, namely "sad or depressed or empty." For respondents that say "no" to E1, a "second chance" question is asked (E2), where we ask about "loss of interest." And for respondents that say "no" to E2, we ask a "third chance" question (E2.1) that asks about being "irritable, grouchy, or in a bad mood."

Assessing symptoms within a Depressive Episode

The symptoms described in E2.1c.1 to E18 must occur during a period of two weeks or longer of feeling depressed, having lost interest in most things, or being irritable or grouchy. They must be present almost every day for at least two weeks. If the respondent's answer suggests the event might have lasted less than two weeks ("Sometimes I do have that problem"), the interviewer should ask "Did that last for a period of two weeks or more?" The symptom must last most of the two week period, but interruptions of one or two days would not negate it if the total time was two weeks or more. Questions asking about thoughts of suicide and suicide attempts have no duration requirements; any occurrence is considered significant.

Change in Status

The symptom questions (E2.1c.1 to E18) in depression refer to a change in the respondent's status. If a person always has the problem and it is always of the same intensity, it does not count as a symptom of a depressive episode.

In some questions (e.g., E10, E15, E15A, E16, E17) the idea of change in status is incorporated into the question. For these, if the respondent says he was "always like that", the interviewer should repeat the question, emphasizing the phrase that indicates change in status.

B. Question-by-Question Specifications

E1   Read the question slowly. It is important to relay to the respondent that the feelings of depression, feeling sad, or empty must have occurred for a period of at least two weeks.

E1c   Read this question slowly and hand Card C to the respondent. It is not necessary to read Card C to the respondent, since the information on the card is also contained in the text of the question. To answer "yes" to this question, the respondent must have had at least one of the symptoms listed on Card C at the same time as the period of depression.

E1c.1   This question asks the respondent to identify the period of depression in the past 12 months that was accompanied by the largest number of problems (from E1c, and listed on Card C). Note that this may not be the two-week period when the respondent felt most severely depressed, or caused the greatest impairment.

If the respondent can't choose one period of depression as having the largest number of problems, the instructions are to ask the respondent to think about the most recent two-week period of depression.

E1d   This question refers to the period identified in the previous question (E1c.1).

E2   This is a "second chance" question for this series.

Read this question slowly, and emphasize the duration of "two weeks or longer." The loss of interest does not have to be absolute, but it must be general. A response like "I quit paying attention to the news" is not sufficient.

E2c   If the respondent does not already have Card C from the E1 series of questions, hand Card C to the respondent at this time. It is not necessary to read Card C to the respondent, since the information on the card is also contained in the text of the question. To answer "yes" to this question, the respondent must have had at least one of the symptoms listed on Card C at the same time as the period of losing interest in things.

E2c.1   This question asks the respondent to identify the period of depression in the past 12 months that was accompanied by the largest number of problems (from E2c, and listed on Card C). Note that this may not be the two-week period when the respondent felt most severely depressed, or caused the greatest impairment.

If the respondent can't choose one period of depression as having the largest number of problems, the instructions are to ask the respondent to think about the most recent two-week period of depression.

E2.1   This is the "third chance" question for the major depression section. The E3 series of questions will be asked if the respondent answered "no" to E1 or E2, or did not meet full criteria at some point in the E1 or E2 series. The E3 series asks the same set of questions as E1 and E2, with the frame of reference "feeling irritable or grouchy or in a bad mood."

Read this question slowly. Emphasize "most of the time" when asking about feelings of irritability, grouchiness, or bad mood. It is not uncommon for a respondent to answer this third chance and continue with the remainder of the depression section.

E2.1c   If the respondent does not already have Card C from the E1 or E2 series of questions, hand Card C to the respondent at this time. It is not necessary to read Card C to the respondent, since the information on the card is also contained in the text of the question. To answer "yes" to this question, the respondent must have had at least one of the symptoms listed on Card C at the same time as the period of irritability.

E2.1c.1:   This question asks the respondent to identify the period of depression in the past 12 months that was accompanied by the largest number of problems (from E2.1c, and listed on Card C). Note that this may not be the two-week period when the respondent felt most severely depressed, or caused the greatest impairment.

If the respondent can't choose one period of depression as having the largest number of problems, the instructions are to ask the respondent to think about the most recent two-week period of depression.

E4   This question asks about having less appetite than usual during the two week period of depression with the largest number of problems listed on Card C.

E5   This question asks about losing weight without trying to. If the respondent volunteers (he/she) was on a diet or was trying to lose weight, code "no."

E5a   A respondent may say that (he/she) did lose weight but does not know exactly how much weight was lost in that specific two-week period. The respondent may only know over the course of a year how much weight (he/she) lost. Probe with: "What is your best estimate?" If the respondent really cannot estimate an approximate number of pounds, code DK: 998 and record any additional information in the comment screen.

E6   This question asks about having a larger appetite than is usual (change in status) during the two-week period.

E7   If the respondent volunteers (he/she) gained weight due to pregnancy or regaining weight loss, code "no."

E7a   A respondent may say that (he/she) did gain weight but does not know exactly how much weight was gained in that specific two-week period. The respondent may only know over the course of a year how much weight (he/she) gained. Probe with: "What is your best estimate?" If the respondent really cannot estimate an approximate number of pounds, code DK: 998 and record any additional information in the comment screen.

E8   Note that any of three kinds of sleeping trouble qualifies in this question: trouble falling asleep, waking in the middle of the night, or waking up too early. At least one of the three has to occur for two weeks, but it does not have to be the same one every night.

E8a   Emphasize the words <u>every day</u> when reading this question. If the respondent says (he/she) woke up two hours before (he/she) wanted to on some days during the two-week period (but not every day), the answer should be coded "no."

E9   The frame of reference for this question is "almost every day," as defined by the respondent.

E12b   The interviewer must probe "Any other reason?" until no other reasons given. Record the respondent's answer verbatim.

E12c   The symptom being assessed is feelings of worthlessness or excessive or irrational guilt. We need to know if these feelings are caused <u>only</u> by the respondent's distress over the impairment due to the episode of depression. For example, if the respondent says, "I felt worthless because I was so tired all the time that I couldn't get my work done", code "yes." However, if the respondent says, "I felt like my whole life was pointless and that I had always been a burden on my family" or "I thought I was to blame for my brother's suicide because I had been angry with him," the answer would be coded "no."

E15   The interviewer should emphasize the phrases <u>a lot more</u> and <u>than is normal for you</u>. If the respondent often has trouble concentrating and never had two weeks when the trouble was worse

than usual, the interviewer should code "no" because there has not been a change in status of sufficient duration.

E22   If the respondent says that it occurred in the opposite order, (that (he/she) felt good when (he/she) woke up, but felt bad after the day went on), code "no" and note the response in the comment box.

E23   If the respondent indicates (he/she) is not currently sexually active, repeat the question emphasizing <u>your interest</u> in sex.

E24   If the respondent says, "I don't know, because nothing good happened during those two weeks," code "no."

E66   Read carefully and emphasize weeks as the measurement respondent should come up with. The respondent is asked to think of the time in the past 12 months (he/she) was depressed and had some of the problems listed. If the respondent gives the duration of a single symptom (eg., I couldn't sleep for 16 months"), (he/she) has missed the intent of the question. The question should be repeated emphasizing the <u>other problems</u>.

    If the respondent answers "about two weeks," probe to make sure it is 14 days. If the answer is 14 days, code "02" and if the answer is between 7-13 days, code "01." For partial weeks, round <u>down</u> (e.g., 3 ½ weeks is coded "03").

E24b   Record the length of time the period of (SX) has been going on.

E24g   If more than one person died, code the first person the respondent mentions.

E26h   If more than one person died, code the first person the respondent mentions.

E25c   If more than one person died, code the first person the respondent mentions.

E25k   If more than one person died, code the first person the respondent mentions.

E26m   This is an open-ended question allowing the respondent to explain any other events that happened that could have caused depressive periods to begin. Record the respondent's answer verbatim, and probe for clarification as needed.

E28   Start of Probe Flow section.

E29   The onset question should be asked by the interviewer emphasizing <u>exact</u> and <u>very first</u>. The question refers to the whole episode (feeling and problems), not individual symptoms.

E29.1   This question is asked if the respondent does not remember how old (he/she) was the very first time (he/she) had a period of this sort. The respondent is asked to estimate about how old (he/she) was the first time (he/she) had a period of depression of this sort.

E29.2   This question is very similar to E29. There is a subtle difference (E29 asks the respondent to recall (his/her) exact age at (his/her) first period of depression; and E29.2 asks the respondent to recall the earliest age (he/she) can clearly remember such a period). It is possible that the respondent is aware of having periods of depression at a very young age, but does not remember them clearly.

Quality Assurance Policies, Standards, and Processes for Ensuring the Quality of Information Disseminated to the Public

NCHS is recognized as one of the major statistical agencies in the Federal Government. In developing its statistical and analytic information products, NCHS recognizes that the quality assurance process begins at the inception of the information development process and routinely employs a number of widely accepted methods and procedures for ensuring quality, including independent assessments of statistical methodologies and observance of professional standards. In order to ensure the utility of its statistical and analytic information products, NCHS conducts independent research and consults with experts in areas such as data collection, data analysis, and a variety of substantive topics and issues. Additionally, NCHS maintains ongoing contact with users, and participates in conferences, workshops, etc., in order to objectively assess and identify the current and future data needs of NCHS constituents. NCHS reviews the quality (including the objectivity, utility, and integrity) of information before it is disseminated and treats information quality as integral to every step of the development of information, including its creation, collection, maintenance and dissemination. Further, NCHS employs a wide variety of dissemination mechanisms to make its statistical and analytic information products widely available and broadly accessible.

In order to assure the objectivity of its statistical and analytic information products, i.e., that they are accurate, reliable, and unbiased, NCHS obtains these data through accepted statistical theory and practice. Dissemination of data also follows generally recognized guidelines in terms of defining acceptable standards regarding minimum response rates, maximum standard errors, cell size suppression, quality of coding and other processing operations. NCHS also maintains staff expertise in areas such as concept development, survey planning and design (including questionnaire development and testing), data collection, data processing and editing, data analysis, evaluation procedures, and methods of dissemination. Further, NCHS strives to demonstrate in its Paper Reduction Act (PRA) clearance packages that each draft information collection will result in information that will be collected, maintained, and used in a way that is consistent with OMB, HHS, CDC, and NCHS information quality guidelines.

NCHS assures the security of its statistical and analytic information products through the enforcement of rigorous controls that protect against unauthorized access to the data, revision or corruption of the data, or unauthorized use of the data. Some of the major controls used at NCHS include access control, user authentication, encryption, access monitoring, provision of unalterable electronic content, and audit trails.

All NCHS statistical and analytic information products undergo a formal clearance process before dissemination. Publications and reports, whether in electronic or paper form, are reviewed by a designated official within the author's office or division and by the NCHS Associate Director for Science (ADS). These reviews cover the clarity of descriptive text, the appropriateness of the methodology, the soundness of the analysis, the adherence to confidentiality and disclosure avoidance restrictions, the readability of tabular and graphic presentations of data, etc. Finally, all products undergo editorial review (e.g., formatting, proofreading, spell checks, proper punctuation, etc.,) In addition, all public-use tapes are reviewed for accuracy and appropriate confidentiality protections. Oral presentations are subject to appropriate supervisory review.

NCHS statistical and analytic information products are derived using generally acceptable statistical practices and methodologies, which are well documented and available to the public. These procedures enable responsible statisticians and analysts outside of NCHS to replicate the NCHS statistical methods and obtain results consistent with those obtained by NCHS.

APPENDIX III

<center>NHANES Analytic Guidelines</center>

<center>June 2004 Version</center>

Beginning in 1999, the National Health and Nutrition Examination Survey (NHANES) became a continuous, annual survey rather than the periodic survey that it had been in the past. For a variety of reasons, including disclosure and reliability issues, the survey data are released on public use data files every two years. Thus, the data release cycle for the ongoing (and continuous) NHANES is described as NHANES 1999-2000, NHANES 2001-2002, NHANES 2003-2004, etc.

Beginning in 2003, the survey content for each two year period is held as constant as possible so as to be consistent with the data release cycle. This was not always the case in the first four years of the continuous survey and some special data release and data access procedures were developed and used for selected survey content collected in "other than two-year" intervals. In addition to the analysis of data from any two year cycle, it is possible to combine two or more "cycles" (e.g. 1999-2000 and 2001-2002) to create NHANES 1999-2002, thus increasing sample size and analytic options. In order to produce estimates with greater statistical reliability, combining two or more 2-year cycles of the continuous NHANES is encouraged and strongly recommended. The user should verify that data items collected in all combined years were comparable in wording and methods.

These analytic guidelines are the most current recommendations from the National Center for Health Statistics and the NHANES Program for use with the NHANES data from the continuous survey. For the most part, the recommendations provided herein are also appropriate for the past (periodic) NHANES surveys. Although

similar in many ways to the previously provided NHANES III analytic guidelines

(http://www.cdc.gov/nchs/data/nhanes/nhanes3/nh3gui.pdf) and the NHANES

1999-2000 Addendum to the NHANES III Analytic Guidelines

(http://www.cdc.gov/nchs/data/nhanes/guidelines1.pdf) , there are a number of

modifications and these latest guidelines should be used for all future analyses of the

continuous NHANES survey and for comparisons with previous NHANES surveys.

These guidelines will be updated on a periodic basis as more information is learned from

the analyses of the NHANES survey data and as new and important statistical procedures

are developed for use in complex surveys like NHANES. Users should regularly visit the

NHANES website to see if a new version of these analytic guidelines has been released.

At this time, with the release of data from NHANES 1999-2000 and NHANES

2001-2002, the recommended procedure for analysis is to consider the time period 1999-

2002 as one survey. Data files for 1999-2000 (interview sample size 9,965 and MEC

examined sample size of 9,282) and 2001-2002 (interview sample size 11,039 and MEC

examined sample size of 10,477) should be concatenated to form a single analytic file

(interview sample size of 21,004 and MEC examined sample size of 19,759).

When analyzing the combined four year data set, the correct sampling weights

must be used to produce unbiased estimates. Unfortunately, there is no simple procedure

for combining the 1999-2000 and 2001-2002 sample weights (like dividing each

individual sample weight by two and using that number as the four-year sample weight).

The sample weights for NHANES 1999-2000 were based on population estimates

developed by the Bureau of the Census before the Year 2000 Decennial Census counts became available. The two-year sample weights for NHANES 2001-2002 are based on population estimates that incorporate the year 2000 Census counts. The two population estimates are not comparable.

Therefore, to facilitate analysis of these first two cycles of the continuous NHANES, the appropriate four-year sample weights have been calculated and added to the demographic data files for both 1999-2000 and 2001-2002. Thus, users of the earlier release of the NHANES 1999-2000 demographic file must use the updated demographic file to appropriately analyze the combined four-year data. These four-year sample weights have the same variable name in each two-year demographic file. For example, for the sample persons for whom there are MEC data items, the variable name for the four year weight is WTMEC4YR.

Income and Poverty Income Ratio (PIR) have also been added to both the NHANES 1999-2000 and NHANES 2001-2002 demographic data files. This is an additional reason to utilize the recently updated (May 2004) demographic files for both two-year time periods. In the future, additional variables (e.g. marital status) will be added to these demographic files, so users should periodically check the NHANES website for notice of such updates.

In addition, the procedure for variance estimation (sampling errors) has been changed. When NHANES 1999-2000 data were released as public use files,

confidentiality and disclosure avoidance principles prohibited the release of a PSU

variable (as was done in past NHANES). A new approach was needed. For NHANES

1999-2000, 52 replicate weights were produced and a jackknife technique recommended

for variance estimation. This procedure is less feasible for a four-year data set or for

multiple combinations of various two-year NHANES data sets. An alternative method

for variance estimation that protects confidentiality and allows the use of "PSU's" was

developed and is now the recommended approach for analysis on the ongoing and

continuous NHANES. This method creates Masked Variance Units (MVU's) which can

be used as if they were Pseudo-PSU's to estimate sampling errors (similar to past

NHANES). The Pseudo-PSU's on the data file are not the "true" design PSU's. They

are a collection of secondary sampling units aggregated into groups (called Masked

Variance Units) for the purpose of variance estimation. They produce variance estimates

that closely approximate the variances that would have been estimated using the "true"

design variance estimates. These MVU's have been created for both NHANES 1999-

2000 and NHANES 2001-2002 and added to the demographic data files for both two-

year periods. They can also be used for the combined four-year data set.

The stratum variable is SDMVSTRA and the PSU variable is SDMVPSU.

Software such as SUDAAN, STATA and SAS can be used to estimate sampling errors by

the Taylor series (linearization) method. Typically, the data set should first be sorted by

SDMVSTRA and SDMVPSU. There are no replicate weights provided for NHANES

2001-2002. Replication techniques can still be used to estimate sampling errors if the

software, such as WESVAR, computes its own set of replicate weights based on the nested PSU within stratum design.

Some data components of NHANES are only available for 1999-2000 or 2001-2002. For these components, the two-year sample weights and the two-year MVU's can be used for analysis. For 2001-2002, the two-year weights and MVU's are provided on the demographic data file. For 1999-2000, the previously released demographic file has been updated to add the MVU's and four-year sample weights. At this time, the preferred approach for calculating sampling errors is to use the MVU's and to ignore the JK-1 technique utilized as an interim approach with the release of the NHANES 1999-2000 data.

On occasion, there may be a particular issue that requires comparison of results from NHANES 1999-2000 with NHANES 2001-2002. For summary statistics such as means and proportions, the appropriate two-year sample weights and MVU's can be used for reasonably valid inferences (although caution should be used when producing estimates for any detailed population subgroup). Calculation of totals (e.g. estimates of the number of persons with a particular characteristic such as BMI greater than 30 or with impaired glucose tolerance) is not appropriate for NHANES 1999-2000 unless the numbers are ratio-adjusted to population counts based on year 2000 Census figures. This is not a issue for NHANES 2001-2002 which was linked to the 2000 Census counts.

To summarize, there are a number of changes and updates to previous NHANES Analytic Guidelines and these revised recommendations should be used for all analyses of NHANES 1999-2002 data. They are also generally applicable to previous NHANES surveys. NHANES 1999-2000 and NHANES 2001-2002 data files should be combined and analyzed as a four-year data set where possible. The demographic file for NHANES 1999-2000 was updated with four-year weights and MVU's (in addition to the previously existing two-year weights). The NHANES 2001-2002 demographic file contains 2-year and 4-year weights along with MVU's. The 4-year weights and MVU's must be used when the two data sets are merged to create an NHANES 1999-2002 data file. With the four-year combined file, one should ignore the two-year sample weights and the 1999-2000 JK-1 replicate weights. Finally, additional information and recommendations will be added to these guidelines on a periodic basis. Users are encouraged to check the NHANES website on a regular basis to be aware of the latest version of these NHANES analytic guidelines.

APPENDIX IV

Weighted Goodness of Fit Test

A weighted goodness of fit test was necessary considering that NHANES 1999-2000

oversampled for minorities and age groups. This enhances the purpose of a goodness of fit test

in determining the adequacy of the statistical model, and assessing how much the model

departures from the observed data. Unlike the traditional goodness of fit test based on

residuals, the weighted variant uses a data grouping strategy that sorts data in deciles

according to estimated probabilities. Each decile includes approximately equivalent sampling

weights that better help estimate the F-adjusted mean residual test [1].

1. Archer KJ, and Lemeshow S. Goodness-of-fit test for a logistic regression model fitted

    using survey sample data. S.J. 2006; 6(1): 97-105.