Folate and vitamin B-12 biomarkers in NHANES: history of their measurement and use[1–5]

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ABSTRACT

NHANES measured folate and vitamin B-12 status biomarkers, starting with serum folate from NHANES I (1974–1975) through 2010. Subsequent NHANES measured additional biomarkers [eg, red blood cell folate, serum vitamin B-12, total homocysteine (tHcy), methylmalonic acid, serum folic acid, and 5-methyltetrahydrofolic acid]. Examples of the uses of these data are wide ranging and include public policy applications, the derivation of reference intervals, and research. Periodically, the National Center for Health Statistics and its federal partners convene expert panels to review the use of the folate- and vitamin B-12–related biomarkers in NHANES. These panels have evaluated the need for results to be comparable across time and with published data and the use of crossover studies and adjustment equations to ensure comparability. With the recent availability of reference methods and materials for serum folate and tHcy, NHANES has started to use traceability approaches to enhance the accuracy and comparability of its results. A major user concern over the years has been the use of cutoffs to estimate the prevalence of inadequate folate and vitamin B-12 status. Because these cutoffs depend on the measurement procedure, several expert panels suggested approaches for dealing with cutoff challenges. This review summarizes the history and use of folate- and vitamin B-12–related biomarkers beginning with NHANES I (1974–1975) through 2010. *Am J Clin Nutr* 2011;94(suppl):1S–10S.

INTRODUCTION

NHANES provides nationally representative information on the US population's health and nutritional status (1–3). The survey collects information with the use of interviews, medical and physical examinations, and clinical tests under standardized conditions through home interviews and at mobile medical examination centers. The Centers for Disease Control and Prevention (CDC) conducted these surveys periodically between 1974 and 1994 (NHANES I in 1974–1975; NHANES II in 1976–1980; and NHANES III in 1988–1994) and has conducted them continuously since 1999. The findings provide the basis for national reference standards for measurements such as height, weight, and blood pressure. Researchers also use these data to assess the prevalence of major diseases and inadequate or excessive nutritional status among US population groups and to identify correlates of these disease and health risks.

The CDC's National Center for Health Statistics (NCHS) is the agency responsible for NHANES. Interrelated changes in science, user needs, and population-based environments occur concurrently but independently across survey time spans. The NCHS must therefore update and revise NHANES content and measurement procedures frequently and in a manner that addresses user needs while it maintains sound science within the survey's mission, resource, and logistic limits. To help with this effort, the NCHS and the Office of Dietary Supplements of the National Institutes of Health recently convened a roundtable expert panel to review the scientific and public health issues involved in past, current, and future measurement of folate and vitamin B-12 status biomarkers in NHANES. Other articles in this supplement discuss the results of the roundtable dialogue (4, 5) and provide an overview of the various roundtable components (6). The current article describes the history and use of these biomarkers in past and current NHANES. When we present serum and red blood cell (RBC) folate data in this article, we express values in nmol/L (1.0 ng/mL = 2.266 nmol/L).

HISTORY OF FOLATE- AND VITAMIN B-12–RELATED BIOMARKERS IN NHANES

Nutritional status biomarkers are important for the assessment of population groups' nutritional status. The classic folate status biomarkers include serum and RBC folate (7, 8). The vitamin B-12 status biomarkers include serum vitamin B-12 and methylmalonic acid (MMA) (7, 9). Total homocysteine (tHcy) is a nonspecific indicator of vitamin B-12 and folate inadequacies (7, 9). Serum folate and vitamin B-12 are direct measures of circulating blood concentrations, and RBC folate reflects intakes over the past 90–120 d (4, 7). MMA and tHcy are functional indicators of status because they measure metabolites that accumulate as a result of deficiencies in the vitamins that their

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metabolism requires (7, 9). Recently, researchers have measured serum folic acid (FA) concentrations (also referred to as serum unmetabolized FA in some cited references) because of concerns that increased exposures to the synthetic FA in fortified foods and dietary supplements may elevate serum concentrations of this folate vitamer and that this may raise safety concerns (10–14).

NHANES has a long history of measurement of folate- and vitamin B-12–related biomarkers (**Table 1**). The first NHANES (1974–1975) measured serum folate, and NHANES II (1976–1980) added RBC folate and serum vitamin B-12 measurements. Measurement of the 2 folate variables has continued through 2010. Various NHANES have measured serum vitamin B-12, tHcy, MMA, and folate vitamers (5-methyltetrahydrofolate and FA) for several survey periods. NHANES 2007–2010 did not measure serum vitamin B-12 and tHcy, and NHANES did not measure MMA in 2005–2010.

The reasons for the addition of the different folate- and vitamin B-12–related biomarkers to NHANES have varied as public health concerns changed and measurement techniques improved. NHANES I (1974–1975) and II (1976–1980) initially measured serum and RBC folate to provide an interpretive aid in the evaluation of abnormal hematologic indexes (15). An expert panel that reviewed NHANES II nutritional biomarker data in 1984 found that the availability of data on these 2 biomarkers offered an opportunity to examine the prevalence of persons at risk of inadequate folate status (15). Subsequently, interest in monitoring the folate status of US population groups increased because of the opportunity to assess status changes associated with the 1998 implementation of the FA fortification program (16). In the postfortification era, the focus of the interest in monitoring folate status has shifted from inadequacy to the safety of excessive intakes (4).

NHANES II (1976–1980) was the first NHANES to measure serum vitamin B-12. At that time, the measurement procedure for serum and RBC folate changed from a microbiological assay (MA) to the Bio-Rad Quantaphase I competitive protein binding measurement procedure (Bio-Rad Laboratories, Hercules, CA) (Table 1). This kit allowed measurement of both serum vitamin B-12 and folate. Subsequently, interest increased in monitoring vitamin B-12 status following the implementation of FA fortification in 1998

TABLE 1
History of measurement of indicators of folate and vitamin B-12 status in NHANES[1]

Measure	Measurement procedure	Matrix	Laboratory	Population age group
Serum and RBC folate				
NHANES I (1974–1975)	MA	Serum	NCEH	25–74 y
NHANES II				
1976–1978	MA	Serum, WB	NCEH	6 mo–74 y
1978–1980	Bio-Rad I	Serum, WB	NCEH	6 mo–74 y
NHANES III				
1988–1991	Bio–Rad I	Serum, WB	NCEH	≥4 y
1991–1994	Bio-Rad II	Serum, WB	NCEH	≥4 y
NHANES 1999–2002	Bio-Rad II	Serum, WB	NCEH	≥3 y
NHANES 2003–2006	Bio-Rad II	Serum, WB	NCEH	≥1 y
NHANES 2007–2010	MA	Serum, WB	NCEH	≥1 y
	LC-MS/MS	Serum, WB	NCEH	One-third subset, ≥1 y
FA and 5-MTHF				
NHANES 1999–2002	Affinity/HPLC	Surplus serum	Tufts	≥60 y
NHANES 2007–2010[2]	LC-MS/MS	Serum, WB	NCEH	One-third subset, ≥1 y
Serum vitamin B-12				
NHANES II (1976–1980)	Bio-Rad I	Serum	NCEH	6 mo–74 y (subset)
NHANES III (1991–1994)	Bio-Rad II	Serum	NCEH	≥4 y
NHANES 1999–2002	Bio-Rad II	Serum	NCEH	≥3 y
NHANES 2003–2006	Bio-Rad II	Serum	NCEH	≥1 y
Total homocysteine				
NHANES III (1991–1994)	HPLC-FD	Surplus serum	Tufts	≥12 y
NHANES 1999–2001	FPIA IMx	Plasma	NCEH	≥3 y
NHANES 2002–2004	FPIA AxSYM	Plasma	NCEH	≥3 y
NHANES 2005–2006	FPIA AxSYM	Plasma	NCEH	≥20 y
Methylmalonic acid				
NHANES III (1988–1994)	GC/MS	Surplus serum	Tufts	30–39 y, ≥65 y
NHANES 1999–2004	GC/MS	Plasma	NCEH	≥3 y

[1] RBC, red blood cell; MA, microbiological assay; NCEH, National Center for Environmental Health, Centers for Disease Control and Prevention; WB, whole-blood lysate; Bio-Rad I, Bio-Rad Quantaphase I commercial kit (Bio-Rad Laboratories, Hercules, CA); Bio-Rad II, Bio-Rad Quantaphase II commercial kit; LC-MS/MS, liquid chromatography–tandem mass spectrometry; FA, serum folic acid (also referred to as serum unmetabolized folic acid); 5-MTHF, 5-methyltetrahydrofolic acid; Tufts, Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston, MA; GC/MS, gas chromatography/mass spectrometry; HPLC-FD, HPLC-fluorescence detection; FPIA IMx, commercial fluorescence polarization immunoassay reagent set (Abbott Laboratories, Abbott Park, IL) on the Abbott IMx analyzer; FPIA AxSYM, commercial fluorescence polarization immunoassay reagent set (Abbott Laboratories) on the Abbott AxSYM analyzer.

[2] Includes measurement of other folate vitamers, in addition to FA and 5-MTHF.

because of concerns that excessive FA intakes might adversely affect vitamin B-12 status (16). Post–FA fortification measurement of serum vitamin B-12 continued until NHANES 2006; NHANES subsequently stopped the measurement of serum vitamin B-12 because the manufacturer stopped production of the Bio-Rad kit, and serum vitamin B-12 concentrations had not shown any appreciable changes in NHANES surveys conducted between 1991 and 2006 (4).

Researchers at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University initially measured tHcy, MMA, and folate vitamers with the use of stored surplus sera from prior NHANES as special research projects (Table 1) (12, 17, 18). After the researchers established these biomarkers' measurement capability for large surveys such as NHANES, and because of public health priorities for monitoring the US population's folate and vitamin B-12 status, several NHANES survey periods subsequently included these biomarkers, and the CDC's National Center for Environmental Health (NCEH) generated these data (Table 1).

Periodically, the NCHS and its federal partners convene expert panels to review the quality of the measurement procedures and the interpretation of NHANES nutritional status data (15, 19, 20). The recent roundtable panel continued this process for folate and added a review of vitamin B-12–related biomarkers (4, 5).

USES OF NHANES

A range of policy, research, industry, and consumer groups uses NHANES data for a variety of applications. We provide examples of several key applications below.

Public policy applications

A highly visible and major use of NHANES nutritional data is to support the development and evaluation of public health policies and programs. The inclusion of folate and vitamin B-12 status indicators in NHANES before and after the 1998 FA fortification policy's implementation is an excellent case study of the use of NHANES data for public health policy decisions (16, 21, 22). The NHANES distribution data for folate- and vitamin B-12–related biomarkers provided useful baseline and time-trend information for the development and monitoring of the FA fortification program. The NHANES data showed marked shifts in the distributions of serum (**Figure 1**A) and RBC folate (Figure 1B), but not serum vitamin B-12 (Figure 1C), before and after fortification. Post-fortification distributions were relatively stable. Because the fortification program involved the addition of FA, but not vitamin B-12, to enriched foods, these findings are consistent with expectations. The biomarker distributions and their changes over time allow an evaluation of whether the desired improvements in status occurred at the low end of the distribution curve (ie, a decrease of inadequate or low folate status) and of the potential for concern at the high end of the distribution curve (ie, increases in folate values associated with potentially excessive intakes) between 2 time periods.

tHcy, a nonspecific indicator of inadequate folate status, also shifted between the pre- and postfortification time periods (**Figure 2**). tHcy accumulates with folate inadequacy but is relatively stable across normal folate ranges. As a result, the change from before and after fortification is limited to the tail on the right side of the distribution curve, as we would expect with a decrease in the prevalence of low folate status.

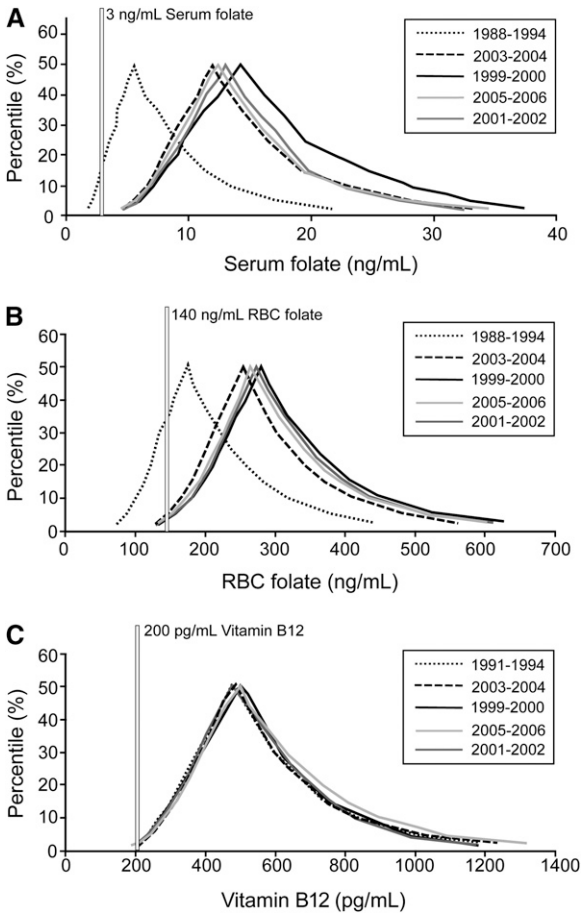

FIGURE 1. Mountain plots of serum folate (A), red blood cell (RBC) folate (B), and serum vitamin B-12 (C). All 3 figures represent the total population of the United States according to NHANES, which spanned 1988–1994, 1999–2000, 2001–2002, 2003–2004, and 2005–2006. The vertical dotted lines indicate commonly used cutoffs for assessment of low serum and RBC folate and serum vitamin B-12. For serum and RBC folate, 1 ng/mL = 2.266 nmol/L. Panels A and B are updated versions of Figure 1 in reference 22, and panel C is an updated version of Figure 2 in reference 21 to include NHANES data published subsequent to these earlier publications (C Pfeiffer, personal communication, 2011).

The combination of data from several national data sets helps one more fully evaluate health changes associated with FA fortification. The FA fortification program's purpose was to decrease the incidence of neural tube birth defects, such as spina bifida (16, 21, 22). The combination of the data on RBC folate from the NHANES with the spina bifida incidence rates from the Vital Statistics data shows the national trends in folate status and spina bifida rates before and after fortification (**Figure 3**).

The discovery of consistent and expected changes in multiple biomarker measures (ie, serum and RBC folate, tHcy, serum vitamin B-12) provides greater confidence than if only one indicator of nutrient status is available. Agencies frequently implement public policy decisions, such as those involved in the design and implementation of the FA fortification program, within a context of considerable uncertainty about the likely magnitude of their effectiveness or the possibility of triggering unintended and unknown safety concerns. Therefore, national monitoring programs such as NHANES are often the best, and in

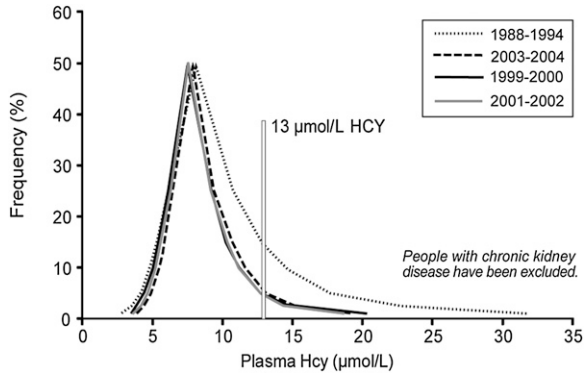

FIGURE 2. Mountain plots of plasma total homocysteine (Hcy) in the total US population (with the exclusion of persons with chronic kidney disease) according to NHANES, which spanned 1991–1994, 1999–2000, 2001–2002, and 2003–2004. This is an updated version of Figure 2 in reference 23 to include NHANES data published subsequent to this earlier publication (C Pfeiffer, personal communication, 2011).

some cases the only, practical way to monitor the population-based effects of national policy interventions (16, 24).

Reference intervals

NHANES data are also useful for the development of population-based reference intervals. Clinical laboratories, health professionals, and researchers use these data as a basis for comparisons to evaluate their own laboratory, patient, or group data. The CDC's growth charts, which the CDC derived from NHANES anthropometric data, are a well-known example of the use of NHANES-supported reference data (25, 26). For folate- and vitamin B-12–related biomarkers, the CDC and others have published reference intervals, beginning with the NHANES III data set (1988–1994) and continuing through recent surveys (18, 22, 23, 27–30). Similar to public policy applications, this application of NHANES data uses both distribution curves and measures of central tendency.

Research and other applications

Researchers frequently use NHANES nutrient biomarker and other data to identify groups at risk of various diseases and health conditions and to determine correlates of disease risk. Researchers use the results for a variety of purposes, such as the generation of hypotheses, the identification of research needs, and the identification of modifiers of nutritional and disease risk.

Studies have used NHANES data to examine relations between folate and vitamin B-12 status biomarkers and a variety of health and disease conditions. These conditions include cardiovascular risk and fitness (31–38), obesity and weight loss (39, 40), lead and neurobehavioral test performance (41), tobacco exposure (42, 43), anemia and cognitive function (12, 44–46), hypothyroidism (47), dental and periodontal health (48, 49), colon cancer (50), and gene-gene and gene-nutrient interactions (51). Evaluations of associations between intakes and/or demographic variables and folate and vitamin B-12 status are also common (10, 52–54). Because results from the NHANES and other observational studies indicated that B vitamin status was associated with a decreased risk of cardiovascular disease, researchers were able to obtain funding for several large randomized clinical trials (55).

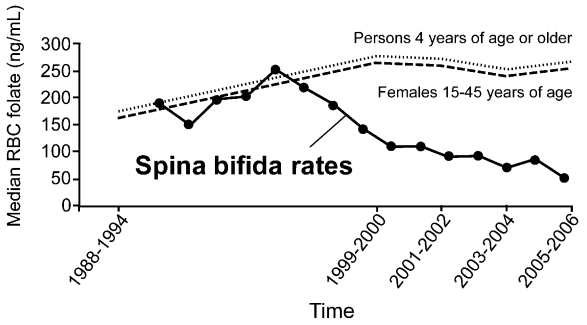

FIGURE 3. Comparison of median red blood cell (RBC) folate values and spina bifida incidence rates that spanned NHANES 1988–1994, 1999–2000, 2001–2002, 2003–2004, and 2005–2006. For RBC folate, 1 ng/mL = 2.266 nmol/L (C Johnson, personal communication, 2011).

COMPARABILITY ISSUES FOR NHANES BIOMARKERS

Now that researchers have >3 decades of experience with NHANES nutrient biomarker data, the comparability of biomarker measurements over time and with published data has been a constant measurement and interpretability challenge for the NCHS and the analytic laboratories that generate the data, as well as for NHANES data users. The recent and evolving availability of reference methods and materials will provide useful tools for tracing results from a given NHANES laboratory or measurement procedure to a commonly accepted reference point, which will provide a basis for marked progress in the resolution of these challenges (4, 5, 56–59).

Comparability of NHANES results across time

NHANES has existed since 1971, NHANES 2011–2012 is currently in the field, and plans for NHANES 2013–2014 are underway. With its long timelines, the survey must constantly adapt to inevitable scientific improvements in the measurement procedures for a given biomarker (Table 1). These changes consistently raise questions about whether differences in biomarker concentrations from one survey period to the next (or even within a single survey period if measurement procedures change or assay drift occurs) are due to methodologic artifacts or reflect true population changes in nutrient status (15, 20–23, 58, 60, 61). The lack of a sound basis for measurement comparability across time jeopardizes a major design component of the NHANES, that of time-trend analyses. The current roundtable reviewed the most recent time-trend challenge, how to deal with differences in results between the Bio-Rad Quantaphase II assay used to measure serum and RBC folates in NHANES 1991–1994 and 1999–2006 and the MA used to measure these biomarkers in NHANES 2007–2010 (4).

When changes in measurement procedures occur, as with folate- and vitamin B-12–related biomarkers (Table 1), NHANES' laboratories commonly conduct crossover studies that measure the same samples with the use of both the "new" and "old" measurement procedures (20–23, 61–63). From the results of these studies, the NCHS can derive statistical equations to adjust (also referred to as "correct" in some publications) the values from one measurement procedure to reflect the values it would have obtained with the second procedure (**Table 2**). In many cases, expert panels have reviewed and concurred with the use of these adjustment equations, and have often noted that time-trend analyses would be

misleading if users did not statistically adjust for measurement procedure differences (4, 5, 15, 20, 58). For example, an expert panel that reviewed the change from the Bio-Rad Quantaphase I to a Bio-Rad Quantaphase II measurement procedure midway through NHANES III (1988–1994) noted differences in serum and RBC concentrations between the 2 laboratory methods (20). The panel recommended the use of a statistical adjustment equation based on a crossover study to adjust the Quantaphase I results to concentrations that would have been obtained with the Quantaphase II procedure (Table 2). The application of this statistical equation to the unadjusted serum and RBC data systematically decreased values across the distribution curve by ≈35% for serum and ≈30% for RBC folate (**Table 3**) (20). In another example, the NCEH and NCHS identified 3 adjustment equations to compare tHcy in NHANES III (1988–1994) and NHANES 1999–2004 data because the 2 surveys used different measurement procedures and laboratories to perform the analyses, as well as a different sample matrix (Tables 1 and 2) (23, 61).

However, in a few cases, experts could not find a statistical model with a good data fit between the measurement procedures of interest (15). For example, an expert panel did not find a satisfactory statistical model to adjust the serum and RBC folate results to convert the MA data to Bio-Rad Quantaphase I procedure data in NHANES II (1978–1980) (15). Instead, the panel recommended merging the data from the 2 measurement procedures for concentrations at the lower half of the distribution curves (<14 nmol/L for serum folate and <340 nmol/L for RBC folate) but not for distributions above these values (Table 2). The panel based this recommendation on the observation that the results of the 2 measurement procedures below these cutoffs were similar but the distributions above these cutoffs diverged substantially. This approach enabled computations of the prevalence of low folate values from the merged data for both measurement procedures, but the merged data could not provide group means. Currently, an evaluation of statistical models to relate the Bio-Rad Quantaphase II serum and RBC folate results from NHANES 1999–2006 to the MA results from NHANES 2007–2008 is underway (62).

In some cases, NCHS has adjusted the NHANES data sets for measurement procedure differences before their release; in other cases, users need to make these adjustments as part of their data analysis (Table 2). Web-based NCHS NHANES documentation and guidance documents are available to inform users about the need to apply a statistical adjustment equation for their time-trend analyses.

Comparability with reference methods and materials ("traceability")

In the development and refining of measurement procedures, laboratory scientists strive for both precision (reproducibility) and accuracy (trueness) (57, 59). Accuracy is the ultimate goal because it incorporates components necessary for both precision and comparability. Scientists evaluate precision by the assessment of variability with the use of replicate measures and monitoring of the stability of quality control pools over time. Information on the precision and other performance characteristics of NHANES measurement procedures for all measured biomarkers, which includes those for folate and vitamin B-12 assessments, is part of the documentation of each NHANES (4, 5).

Accuracy, the ultimate measurement goal, has historically been more difficult to assess than precision (59). The multiple expert panels convened to review folate biomarkers in NHANES have generally considered the MA to be the "gold standard" measurement procedure for serum and RBC folates because it fully measures the multiple forms of folate species that exhibit folate vitamin activity and does not measure folate species that lack vitamin activity (4, 8, 15). The competitive protein binding commercial assay kits have not always measured all biologically active species accurately. Measurement of folate vitamers in a manner that reflects their in vivo concentrations has been a challenge for the more recent liquid chromatography–tandem mass spectrometry measurement procedures because of interconversions and degradation of some of the vitamers during sample handling and processing (4, 8).

NHANES analytic laboratories, along with the broader scientific community, are beginning to define accuracy with the use of a "traceability" process that links the results from an individual sample to a formally accepted, well-documented reference method (57, 59). Traceability is independent of the measurement system, location, or time of analysis. The National Institute of Standards and Technology (NIST) and the NCEH have received formal recognition for their measurement procedures for serum 5-methyltetrahydrofolate, FA, and tHcy as reference methods from the Joint Committee on Traceability in Laboratory Methods (64). The NIST has also made available standard reference materials with certified values for serum 5-methyltetrahydrofolate and tHcy, reference values for serum FA, and method-specific information values for total serum folate and serum vitamin B-12 (65). The NIST is developing a standard reference material for plasma MMA and serum vitamin B-12 (5).

When the serum-based NIST Standard Reference Materials 1955 became available in 2006, NCEH was able to compare results from the NHANES Bio-Rad Quantaphase II procedure with the NIST information values for serum total folate by liquid chromatography–tandem mass spectrometry and MA. The Bio-Rad results were 25–40% lower (4). The National Institute for Biological Standards and Control has a reference material for RBC folate (95/528) with consensus values from various MA and protein binding measurement procedures. The Bio-Rad results on this material were 45% lower than the consensus value (4). Thus, the availability of external reference materials showed that the Bio-Rad procedures raised accuracy concerns. With conversion to the MA in 2007, NCEH results for serum folate were similar to the assigned values for the reference materials. To ensure that measurement procedures do not shift over time, NCEH has continuously used available reference materials for periodic checks, so-called calibration verifications. As mass-spectrometry-based measurement procedures become more available for routine use, the NCEH is incorporating higher-order reference measurement procedures into NHANES analytic procedures.

Comparability of NHANES results with other published data

NHANES data users frequently need or want to compare NHANES results with other published data. These comparisons require an understanding of the comparability of results from different measurement procedures, and this information is often not available. For example, Statistics Canada recently released the results of its Canadian

TABLE 2
Adjustment equations to allow comparison of folate- and vitamin B-12–related biomarker results across NHANES when measurement procedure changes occurred[1]

Measure	Survey	Conversion	Measurement procedure adjustment	Who?[2]	References
Serum folate	NHANES II 1978–1980	MA to Bio-Rad I	To obtain a national probability sample, merge the 2 data sets only for values <6 ng/mL (14 nmol/L);[3] for values ≥6 ng/mL, data sets cannot be merged and nationally representative means cannot be calculated	User	(15)
	NHANES III 1988–1994	Bio-Rad I to Bio-Rad II	Adjusted value (Bio-Rad I) = −0.1411 + 0.6849 × (unadjusted value Bio-Rad I)	NCHS	(20) and (28)
	1991–2010	Bio-Rad II to MA	Adjustment information in press	User	(61)
RBC folate	NHANES II	MA to Bio-Rad I	To obtain a national probability sample, merge the 2 data sets only for values <150 ng/mL (340 nmol/L)[3]	User	(15)
	1978–1980		For values ≥150 ng/mL, data sets cannot be merged and nationally representative means cannot be calculated	—	—
	NHANES III 1988–1994	Bio-Rad I to Bio-Rad II	Adjusted value (Bio-Rad I) = −0.1411 + 0.6849 × (unadjusted value Bio-Rad I)	NCHS	(20) and (28)
	1991–2010	Bio-Rad II to MA	Adjustment information in press	User	(61)
Serum vitamin B-12	1976–1980 compared with 1991–2006	Bio-Rad I to Bio-Rad II	No adjustment needed		
Total homocysteine	1991–1994	Tufts HPLC serum to Tufts HPLC plasma	Log_{10} Tufts HPLC plasma = 1.042 × log_{10} Tufts HPLC serum – 0.079; r = 0.98; 95% CI for slope: 0.957, 1.126; 95% CI for intercept: −0.156, −0.002	User	(23)
		Tufts HPLC plasma to NCEH HPLC plasma	Log_{10} NCEH HPLC = 1.165 × log_{10} Tufts HPLC plasma – 0.120; r = 0.97; 95% CI for slope: 1.110, 1.220; 95% CI for intercept: −0.170, −0.070	User	(23)
		NCEH HPLC plasma to AxSYM plasma	Log_{10} AxSYM = 1.104 × log_{10} NCEH HPLC – 0.006; r = 0.99; 95% CI for slope: 0.998, 1.029; 95% CI for intercept: −0.020, 0.0008	User	(23)
	1999–2000 and 2001	IMx to AxSYM	Log_{10} AxSYM = 0.983 × log_{10} IMx + 0.042; r = 0.99; 95% CI for slope:0.969, 0.997; 95% CI for intercept: 0.030, 0.054	User	(23)
	2002 and 2003–2004	AxSYM	No conversion needed	—	—
MMA	1988–1994 to 1999–2004	Tufts serum to NCEH plasma	No formal MMA method comparison was conducted, which precluded direct comparison of the MMA results between these 2 surveys	User	(21)
Serum creatinine[4]	1988–1994 to 1999–2000	Jaffé method (kinetic alkaline picrate) to Roche enzymatic assay traceable to reference measurement procedures	Adjusted serum creatinine (Y) = 0.147 + 1.013 × unadjusted serum creatinine (mg/dL); (slope was not statistically significantly different from 1.0 and intercept was not statistically different from 0)	User	(63)

[1] MA, microbiological assay; Bio-Rad I, Bio-Rad Quantaphase I commercial assay kit (Bio-Rad Laboratories, Hercules, CA); Bio-Rad II, Bio-Rad Quantaphase II commercial assay kit; NCHS, National Center for Health Statistics, the Centers for Disease Control and Prevention; RBC, red blood cell; Tufts, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA; NCEH, National Center for Environmental Health, Centers for Disease Control and Prevention; AxSYM, Abbott AxSYM analyzer (Abbott Laboratories, Abbott Park, IL); ImX, Abbott ImX analyzer (Abbott Laboratories); MMA, methylmalonic acid.

[2] "Who?" refers to whether the statistical adjustment should be done by the user of the NHANES data, or NCHS incorporated the adjustment into the data set prior its public release.

[3] Unit conversion: 1 ng/mL serum or RBC folate = 2.266 nmol/L.

[4] The adjustment equation for serum creatinine is included in this table because this variable is often used in analyses that involve serum vitamin B-12, total homocysteine, and MMA because of its confounding effect on these variables.

Health Measures Survey for 2007–2009 (66). Comparisons of the Canadian 2007–2008 results with NHANES 2003–2006 results for adults aged 20–70 y showed differences in medians for RBC folate (1266 for Canada compared with 603 nmol/L for the United States), plasma tHcy (6.7 compared with 8.0 μmol/L), and serum vitamin B-12 (299 compared with 339 pmol/L) (C Stempos, personal communication, 2011). Health Canada and NCEH used different measurement procedures to obtain their biomarker results. Without direct

TABLE 3
Unadjusted and adjusted serum and red blood cell (RBC) folate values for a sample population of individuals aged 20–39 y in NHANES III (1988–1994)[1]

	Percentiles					
Biomarker	5th	25th	50th	75th	95th	Prevalence
			nmol/L			%
Serum folate						
Unadjusted	6.3	10.2	14.0	21.1	38.3	6.5
Adjusted	4.1	6.6	9.3	14.0	25.8	8.1
RBC folate						
Unadjusted	274	399	508	680	1008	1.9
Adjusted	193	281	360	485	725	4.3

[1] The prevalence of the unadjusted low values for serum and RBC folate concentrations were based on cutoffs for assessment of folate status of <6.8 nmol/L (3 ng/mL) for serum and <227 nmol/L (100 ng/mL) for RBC folate. The prevalence of the adjusted low values is based on cutoffs in which the original cutoffs were adjusted by the same equation as was used for the biomarker concentrations. The cutoffs that resulted were <4.3 nmol/L (1.9 ng/mL) for serum and <155 nmol/L (68.5 ng/mL) for RBC folate. (Conversion: 1 ng/mL = 2.266 nmol/L.) Adapted from reference 20.

comparisons of the measurement procedures from the 2 surveys, or comparable information on the traceability of both procedures to a higher-order reference method, it is not possible to determine whether these differences in folate and vitamin B-12 concentrations between 2 neighboring countries with similar FA fortification programs and food supplies are real or are due to differences in analytic measurement procedures. A recent expert panel found similar challenges in the comparison of Canadian and US results from the 2 surveys for vitamin D biomarker data (67).

Comparability of NHANES results with published data is also important for the use of cutoffs of adequacy to determine the prevalence of at-risk groups. Cutoffs of nutritional status should discriminate accurately between healthy and at-risk groups, with few misclassification errors. However, when one measurement procedure is used to determine cutoffs, and users compare these cutoffs with results from a different procedure, the generalizability of the cutoff value has questionable value (9, 15, 20). Moreover, the selected cutoff can markedly affect prevalence estimates. For example, the NHANES-based prevalence of low serum vitamin B-12 for adults varied from 3% to 25%, and depended on which cutoff the user applied from 3 commonly used cutoffs of vitamin B-12 status (R Bailey, personal communication, 2011).

The appropriateness of commonly used cutoffs for the estimation of the prevalence of at-risk groups from NHANES data has been a consistent focus of expert panels since the first folate-based panel in 1984. Over the years, experts have identified a variety of approaches to address this troublesome issue. A 1984 expert panel examined the effect on reference ranges for serum and RBC folate concentrations after the deletion of data on individuals with parameters that indicated folate deficiency (eg, hemoglobin concentrations, mean corpuscular volume, macrocytosis) to identify serum and RBC folate concentration ranges that differed between supposedly at-risk and folate-replete groups (15). This approach was not useful, perhaps because too few persons had abnormalities in these hematologic parameters to affect the results.

Another expert panel recommended the collection of serum and whole-blood samples from patients with a folate-deficiency diagnosis (eg, marrow abnormalities and response to folacin treatments) and folate-replete populations in a clinical setting. Panel members suggested the inclusion of these blood samples in the NHANES analytic runs to help define a useful cutoff that minimized misclassification of healthy and at-risk NHANES participants (15, 19). Survey logistics precluded implementation of this approach.

For NHANES III (1988–1994), an expert panel recommended handling the method dependence of the cutoff values similarly to the mathematic adjustments that NCHS used to account for differences in results between the Bio-Rad Quantaphase I and II assay kits (20). Specifically, the panel recommended application of the adjustment equation to the selected, commonly used cutoffs, as well as to the NHANES data. This adjustment changed the original cutoffs of adequate folate status from <6.8 to <4.3 nmol/L for serum folate and from <227 to <155 nmol/L for RBC folate. This cutoff adjustment decreased the estimated prevalence of low serum folate concentrations from 26% to 8% and low RBC folate concentrations from 12% to 4% (20).

Selhub et al (30) identified cutoffs by the determination of the inflection point between circulating concentrations of folate or vitamin B-12 (eg, serum and RBC folate, serum vitamin B-12) and their respective functional indicators of status (eg, plasma tHcy and MMA). From their results, they proposed a cutoff value of <10 nmol/L for serum folate and <340 nmol/L for RBC folate based on the lowest plasma tHcy. For serum vitamin B-12, the cutoff would be <150 pmol/L based on the lowest MMA and <300 pmol/L based on the lowest tHcy. Durazo-Arvizu et al (68) used a similar approach to estimate the inflection point between serum 25-hydroxyvitamin D and parathyroid hormone. They showed that the statistical model researchers selected for these types of analyses affected the derived cutoff value.

The preferred approach to the identification of cutoff values is to conduct clinical trials that allow direct comparison of biomarker concentrations with the appearance of adverse clinical signs and symptoms, and in which the measurement procedure is traceable to a higher-order reference method (4, 5, 58). Researchers recognized all of the examples above as surrogates for the preferred method of defining cutoffs of adequacy and/or safety but undertook them because of the absence of more robust data at the time.

Effects of systematic bias

Accuracy is obviously important for NHANES for uses such as derivation of national reference intervals. Moreover, accuracy issues can affect prevalence estimates of at-risk groups, for which many researchers use NHANES data. For example, midway through NHANES III (1988–1994), the NCEH began to use the Bio-Rad Quantaphase II assay kit, instead of the Quantaphase I, to measure serum and RBC folate (20). The systematic difference between these 2 measurements required a statistical adjustment to the Quantaphase I values to make them comparable with the Quantaphase II results. Comparisons of the unadjusted with the adjusted distribution curves for the Quantaphase I results produced differences of ≈35% for serum and ≈30% for RBC folate (Table 3). If unadjusted cutoffs for low folate status (<6.8 nmol/L for serum and <226 nmol/L for RBC folate) had been used in

conjunction with the adjusted biomarker concentrations, prevalence estimates of low serum folate and low RBC folate would have been 26% and 12%, respectively (data not shown). With the use of adjusted cutoff values with the adjusted biomarker concentration data, prevalence estimates of 8% for serum and 4% for RBC folate were obtained (Table 3). The expert panel recommended that the same adjustment be used for both the population-based biomarker data and the cutoffs (20).

Within-person variance

Errors in prevalence estimates also occur when users fail to adjust for within-person variability (WP) in biomarker measurements (69). WP (ie, differences in measurement results within a person between measurement periods) and between-person variation (BP) are part of biological variation. Ideally, users should minimize WP to avoid masking or attenuation of the differences of interest, BP variabilities. Failure to adjust for WP can inappropriately inflate prevalence estimates of nutrient biomarkers. Although WP adjustment has become commonplace in dietary intake estimates, its use with nutrient biomarkers has been rare.

The estimation of WP and BP variations requires replicate measures on given individuals. NHANES III (1988–1994) and NHANES 1999–2002 collected replicate phlebotomies on a subsample of adults (70, 71). The WP-to-BP ratio was <1 for biomarkers of interest in the assessment of folate and vitamin B-12 status (**Table 4**). However, even ratios of this size can affect prevalence estimates (69). Except for serum vitamin B-12, the WP-to-BP ratio is quite similar for a given biomarker between the 2 surveys. With the recent reporting of the NHANES 1999–2002 results on biological variation (71) and the earlier availability of replicate results from NHANES III (1988–1994) (70), it is now possible to consider their appropriateness and use with the folate- and vitamin B-12–related status biomarkers.

TABLE 4
Estimates of the ratio of within-person to between-person error based on exam 1 and exam 2 data from NHANES III (1988–1994) and NHANES 2001–2002[1]

Variable	Sample size	Correlation exam 1 compared with exam 2	Estimated ratio
	n	*r*	
Serum folate (nmol/L)			
NHANES III	2304	0.84	0.192
NHANES 2001–2002	538	0.79	0.261
RBC folate (nmol/L)			
NHANES III	1597	0.96	0.043
NHANES 2001–2002	545	0.94	0.069
Serum vitamin B-12 (pmol/L)			
NHANES III	1138	0.53	0.885
NHANES 2001–2002	539	1.00	0.003
Methylmalonic acid (μmol/L)			
NHANES III	—	—	—
NHANES 2001–2002	546	0.77	0.300
Serum homocysteine (μmol/L)			
NHANES III	866	0.87	0.150
NHANES 2001–2002	547	0.81	0.241
Serum creatinine (mg/100 mL)			
NHANES III	2076	0.95	0.051
NHANES 2001–2002	536	0.95	0.058
Hemoglobin (g/dL)			
NHANES III	2295	0.93	0.076
NHANES 2001–2002	549	0.93	0.070
MCV (fL)			
NHANES III	2295	0.98	0.015
NHANES 2001–2002	549	0.98	0.018

[1] The estimated ratio of within-person variation (WP) to between-person error (BP) was calculated with the use of the formula WP/BP = $(1 - r)/r$ (C Sempos, personal communication, 2011). RBC, red blood cell; MCV, mean corpuscular volume.

SUMMARY

NHANES has measured at least some biomarkers of folate and vitamin B-12 status throughout its history, starting with NHANES I (1974–1975). Researchers and policymakers have used the results for a broad range of policy and research applications. Over the years, several expert panels have evaluated the measurement and use of folate and vitamin B-12 status biomarkers. Key challenges have included the effects of measurement procedures that changed on the comparability of results over time and the appropriateness of adequacy cutoffs for NHANES applications. The measurement of folate and vitamin B-12 biomarkers in future NHANES will benefit from the evolving availability of well-documented reference methods and materials.

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