Overview of a roundtable on NHANES monitoring of biomarkers of folate and vitamin B-12 status: measurement procedure issues1–6

Elizabeth A Yetley, Paul M Coates, and Clifford L Johnson

ABSTRACT
A roundtable dialogue to discuss “NHANES Monitoring of Biomarkers of Folate and Vitamin B-12 Status” took place in July 2010. This article provides an overview of the meeting and this supplement issue. Although the focus of the roundtable dialogue was on the measurement of folate and vitamin B-12 status biomarkers in NHANES, this article also describes the relevance and importance of these issues for clinical and research laboratories. The roundtable identified the microbiological assay (MA) as the gold standard for measurement of serum and red blood cell folate concentrations. The roundtable noted that differences in results between the Bio-Rad Quanaphase II procedure (Bio-Rad Laboratories, Hercules, CA) that NHANES 1991–1994 and 1999–2006 used and the MA that NHANES 2007–2010 used will require adjustment equations to evaluate time trends. The roundtable found that the close agreement between the serum results for the MA and liquid chromatography–tandem mass spectrometry (LC-MS/MS) procedures supported the conversion to LC-MS/MS for serum folate in future NHANES. The roundtable recognized the uncertainty about whether subclinical vitamin B-12 deficiency is a public health concern but encouraged reinstatement of at least one circulating vitamin B-12 measure and one functional vitamin B-12 status measure in future NHANES. The use of serum vitamin B-12 and plasma methylmalonic acid would provide continuity with past NHANES. The roundtable supported the continued use of the National Institute of Standards and Technology (NIST) reference materials in NHANES biomarker analyses and the further development of additional reference materials by the NIST. Am J Clin Nutr 2011;94 (suppl):297S–302S.

INTRODUCTION
Since the early 1970s, NHANES have provided information on the nutritional and health status of the US population (1). Policymakers and researchers use the survey results to inform and evaluate public health programs and policies, develop national reference intervals for nutrient and health indexes, and generate research hypotheses.

Scientists often express NHANES results as point estimates (eg, for prevalence estimates of at-risk groups) and trends over time. Therefore, the surveys require highly accurate measurement procedures to ensure that cutoffs of nutrient adequacy and safety derived from published literature or on the basis of commonly accepted clinical guidelines are appropriate for the NHANES context. Accurate measurement procedures are also necessary to yield trends in estimates over time that can be attributed to real changes in nutritional status and are not simply artifacts of changes in, or problems with, measurement procedures. Given the long and repetitive nature of NHANES, which started in 1971 and is still ongoing, NHANES must also be responsive to changes in the measurement of nutrient biomarkers that inevitably occur as science evolves.

Various NHANES have measured several folate- and vitamin B-12–related biomarkers over the past 3 decades (Table 1). Because of the critical need for rigorous scientific standards for NHANES measures within the context of a science base that changes constantly, sponsoring agencies have periodically convened expert panels to review the measurement of nutrient biomarkers in these surveys (1). On 15–16 July 2010, the Office of Dietary Supplements (ODS) of the National Institutes of Health and the Division of Health and Nutrition Examination Surveys of the National Center for Health Statistics, Centers for Disease Control and Prevention, convened the latest expert group, a roundtable to discuss “NHANES Monitoring of Biomarkers of Folate and Vitamin B-12 Status: Measurement Procedure Issues.” The purpose of this roundtable was to assess measurement procedure issues for the NHANES monitoring of biomarkers of folate and vitamin B-12 status, which included past, current, and future surveys. This overview provides a roadmap for the background presentations.

1 From the Office of Dietary Supplements, National Institutes of Health, Bethesda, MD (EAY, PMC), and the National Center for Health Statistics, Centers for Disease Control and Prevention, Hyattsville, MD (CLJ).
2 The authors dedicate this article and the roundtable to Mary Frances Picciano, who was the driving force behind this effort prior to her death in August 2010.
3 Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the Centers for Disease Control and Prevention (CDC), the National Institutes of Health (NIH), or the US Department of Health and Human Services (DHHS). Certain commercial equipment, instruments, or materials are identified in this article to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the CDC, the NIH, or the DHHS, nor does it imply that the materials or equipment identified are necessarily the best available for this purpose.
4 Supported by the National Center for Health Statistics, CDC, and the Office of Dietary Supplements, NIH.
5 Address reprint requests to PM Coates, Director, Office of Dietary Supplements, National Institutes of Health, 6100 Executive Boulevard, Room 3B01, MSC 7517, Bethesda, MD 20892-7517. E-mail: coatesp@od.nih.gov.
6 Address correspondence to EA Yetley (retired), Office of Dietary Supplements, National Institutes of Health, 6100 Executive Boulevard, Room 3B01, MSC 7517, Bethesda, MD 20892-7517. E-mail: beth@yetley.com.
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TABLE 1

| Biomarker                  | Survey                |
|----------------------------|-----------------------|
| Serum folate               | 1974–75, 1976–80, 1988–94, 1999–2010 |
| RBC folate                 | 1976–80, 1988–94, 1999–2010 |
| Serum FA and 5-MTHF        | 1999–2002, 2007–2010 |
| Serum vitamin B-12         | 1976–80, 1991–94, 1999–2006 |
| Total homocysteine         | 1991–94, 1999–2006    |
| Methylmalonic acid         | 1988–94, 1999–2004    |

1 RBC, red blood cell; FA, folic acid (also referred to as unmetabolized folic acid); 5-MTHF, 5-methyltetrahydrofolinic acid.
2 Based on a special research project using surplus sera that was conducted at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston, MA.
3 Based on a one-third subset research sample and a study conducted by the National Center for Environmental Health at the Centers for Disease Control and Prevention, Atlanta, GA.
4 Results from a special research project at Tufts University that used surplus sera for persons aged ≥12 y.
5 Results from a special research project at Tufts University that used surplus sera for adults aged 30–39 y and ≥65 y.

The 2009 roundtable acknowledged that many clinical and research laboratories lack sufficient funding for, or the analytic skills to use, expensive analytic equipment and to conduct high-order measurement procedures. However, the roundtable also felt strongly that laboratories must be familiar with 1) the urgent need for measurement accuracy, 2) generally recognized performance characteristics, and 3) available guidelines (4).

One of the background articles in this journal supplement provides information on key concepts and guidelines for clinical laboratory testing and how working laboratories that commonly use commercial assay kits or in-house measurement procedures can achieve traceability (5). Other roundtable summaries in this supplement provide information on performance characteristics of the NHANES measurement procedures (2, 3); many of these measurement procedures are also available to research and clinical laboratories. Ultimately, commercial assay kit manufacturers will have primary responsibility for improvement of their kits. However, expert panels such as the 2009 and 2010 roundtables and laboratory scientists who ask manufacturers of commercial kits about their kits’ performance and traceability characteristics will help move the science forward and encourage manufacturers to make necessary improvements.

As with measurement procedures, the issues related to the use and interpretation of biomarkers of folate and vitamin B-12 status also have broad applicability beyond NHANES contexts (1, 6–8). For example, all users of these data need to understand what the different biomarkers reflect or measure. Users also need to understand the derivation of cutoffs of status and to determine the relevance of published or commonly used cutoffs to their own setting.

A major difference between epidemiologic research such as NHANES and the use of folate and vitamin B-12 status biomarkers in clinical settings is that clinical settings can often evaluate biomarker results within the context of other signs and symptoms, whereas NHANES often lacks such information. However, the roundtable noted that identification of subclinical vitamin B-12 deficiency, whether in the clinical setting or in epidemiologic research, depends completely on concentrations of biomarkers, such as serum vitamin B-12, plasma methylmalonic acid (MMA), and total homocysteine (tHcy) because this condition is asymptomatic (3, 7). In a discussion of an example of a difference between epidemiologic and clinical settings, the roundtable members agreed that red blood cell (RBC) folate may not have an advantage over serum folate concentrations for assessment of the folate status of the US population in NHANES. However, the roundtable pointed out that RBC folates are often necessary in clinical settings where recent drastic changes in the folate intakes of ill patients may rapidly affect serum folate concentrations (2).

In this situation, RBC folate would provide a better indication of body stores than would serum folate.

AGENDA

A planning committee (PC) of government scientists who measure and use NHANES folate- and vitamin B-12-related biomarkers and academicians with expertise in the measurement and assessment of folate and vitamin B-12 status developed the roundtable agenda (Table 2) and identified panelists for the roundtable (see Table S1 under “Supplemental data” in the online issue). The PC chose roundtable panelists with a broad range of expertise in, for example, laboratory science, epidemiology, biostatistics, folate and vitamin B-12 assessment, clinical medicine, and NHANES use.
Following the successful formats that prior NHANES-related expert panels have used (1), the PC organized the roundtable meeting agenda to include a combination of background presentations and dialogue sessions. In past NHANES expert panel meetings, the background presentations provided detailed descriptions of the NHANES measurement procedures (ie, quality controls and performance characteristics) and crossover study results, with appropriate adjustment equations when measurement procedures changed. The PC agreed that similar presentations by Christine Pfeiffer of the National Center for Environmental Health (NCEH) at the Centers for Disease Control and Prevention in Atlanta would be essential for the roundtable (Table 2). Pfeiffer and her group have been responsible for all the folate- and vitamin B-12–related biomarker measurements in NHANES, except for several special research projects at Tufts University that used surplus sera from prior surveys. Pfeiffer’s presentations addressed the measurement of serum and RBC folates [which included an interlaboratory study on the use of a microbiological assay (MA) to measure serum and RBC folates (9)], serum vitamin B-12, MMA, and tHcy in various NHANES (Table 1). Because Pfeiffer’s information was the main focus of the roundtable dialogue, the articles that describe the roundtable review summarize these presentations (2, 3).

The PC also benefited from lessons learned from the 2009 roundtable on the measurement of serum 25-hydroxyvitamin D in NHANES (4). Previous expert panels reviewed NHANES measurement procedures in detail, and the 2009 roundtable also reviewed the relevant reference methods and materials that the National Institute of Standards and Technology (NIST) had recently made available or was developing. NHANES recently incorporated NIST reference methods and materials for several folate- and vitamin B-12–related biomarkers as well as for serum 25-hydroxyvitamin D (2–4). The 2009 roundtable found these reference methods and materials to be an invaluable resource for the resolution of longstanding NHANES measurement procedure comparability and accuracy problems (4). The PC, which anticipated a similar need to review these issues by the 2010 roundtable, scheduled presentations by Karen Phinney on the NIST reference materials and methods for serum folates, vitamin B-12, MMA, and tHcy (Table 2). As with the presentations on the NHANES measurement procedures, the articles in this supplement that describe the roundtable’s review include the presentations on the NIST reference methods and materials (2, 3).

The 2009 roundtable experts (4), like their 2010 counterparts (Table S1 available under “Supplemental data” in the online issue), represented a broad range of expertise. Although this range of expertise added useful breadth and provided the opportunity to integrate concepts from a range of scientific perspectives, an assessment of the 2009 roundtable’s discussions found that the group’s dialogue would have benefited from some general background information to facilitate a common understanding of the wide-ranging concepts and terminologies involved in the roundtable review. Therefore, planners of the 2010 roundtable included several background presentations in the agenda. These presentations reflected the individual perspectives of the presenters.

The first 2 background presentations, by Elizabeth Yetley of the ODS and Joseph Massaro of Boston University (Table 2), covered the history and use of folate and vitamin B-12 biomarkers in

### TABLE 2

| Roundtable agenda† | Reference |
|--------------------|-----------|
| **General background information** | | |
| History and use of folate and vitamin B-12 biomarkers in NHANES: implications for NHANES measurements—Elizabeth A Yetley | (1) |
| Statistical approaches of the chemist: implications for NHANES measurements—Joseph Massaro | (1) |
| Metrological principles for standardization of laboratory measurement procedures: implications for measurement of biomarkers of folate and vitamin B-12 in NHANES—John H Eckfeldt | (5) |
| **Folate status biomarkers** | | |
| The history of folate assessment and its implications for measurement of biomarkers of folate status in NHANES—Barry Shane | (6) |
| Measures of serum and RBC folate in NHANES—Christine M Pfeiffer | (2) |
| Comparison of serum and RBC folate MA results, NHANES 2007–2008—Christine M Pfeiffer | (9) |
| Measurement of serum 5-MTHF and FA in NHANES 2001–2002—Jacob Selhub | (10) |
| Measurement of folate vitamers in serum and whole blood with LC-MS/MS—Christine M Pfeiffer | (2) |
| Reference methods, materials, and calibrators for 5-MTHF, FA, total folate, and 5-FTHF in serum—Karen W Phinney | (2) |
| Population monitoring of serum 5-MTHF and FA in NHANES—Regan L Bailey | (11) |
| Roundtable discussion: measurement of total serum and RBC folate, serum 5-MTHF and FA, and other folate vitamers in NHANES | (2) |
| **Vitamin B-12 status biomarkers** | | |
| History of vitamin B-12 assessment and its implications for measurement of biomarkers of vitamin B-12 status in NHANES—Ralph Carmel | (7) |
| Measurement of serum vitamin B-12 in NHANES—Christine M Pfeiffer | (3) |
| Measurement of MMA in NHANES—Christine M Pfeiffer | (3) |
| Reference methods, standards, calibrators for serum vitamin B-12 and MMA—Karen W Phinney | (3) |
| MMA as an NHANES biomarker of vitamin B-12 status—Regan L Bailey | (3) |
| Measurement of holoTC in population-based studies—Ebba Nexo | (8) |
| Roundtable discussion: measurement of biomarkers of vitamin B-12 status in NHANES | (3) |
| Measurement of tHcy in NHANES—Christine M Pfeiffer | (3) |
| Reference materials, reference methods, and calibrators for tHcy in serum—Karen W Phinney | (3) |
| Roundtable discussion: measurement of tHcy in NHANES | (3) |

† RBC, red blood cell; MA, microbiological assay; 5-MTHF, 5-methyltetrahydrofolic acid; FA, folic acid (also referred to as unmetabolized folic acid); LC-MS/MS, liquid chromatography–tandem mass spectrometry; 5-FTHF, 5 formyltetrahydrofolic acid; MMA, methylmalonic acid; holoTC, holotranscobalamin; tHcy, total homocysteine.
NHANES, the key scientific challenges that previous expert panels identified when they examined folate-related biomarkers in NHANES, and statistical issues of key importance to NHANES users. The article by Yetley and Johnson (1) summarizes these presentations. Comparability of measurement procedures across survey periods and the relevance of commonly used status cutoffs to the NHANES context have been a persistent problem for past expert panels, and the PC anticipated that these issues would also challenge the 2010 roundtable. In addition, the PC thought that the roundtable should be aware of the statistical challenges involved in ensuring that biomarker measurements are accurate at the tails of the biomarker distributions as well as at the means.

Because of the availability and development of NIST reference methods and standards and the usefulness of these standards for NHANES biomarker data that the 2009 roundtable had identified (4), the PC scheduled a presentation by John Eckfeldt of the University of Minnesota. Eckfeldt discussed the evolving concepts and use of traceability approaches by clinical laboratories to ensure accuracy and comparability of results across time, laboratories, and measurement procedures (Table 2) (5). These concepts were unfamiliar to many panelists on the 2009 roundtable but played a critical role in the group’s review and conclusions. Therefore, the PC deemed the inclusion of this background information to be essential for the 2010 roundtable.

Another lesson learned from the 2009 roundtable was the need for background information on the history of the measurement and assessment of folate and vitamin B-12 status. Commonly used cutoffs of adequacy depend on the measurement method, so understanding their derivation was important for the roundtable’s evaluation of current and future NHANES measurement procedures. Moreover, as measurement procedures have evolved, experts have obtained insights into measurement accuracy and potential biases or interferences. Understanding what the various biomarkers measure and the relevance of the approaches that experts have used to derive cutoffs of adequacy can inform an evaluation of their usefulness for future NHANES. To provide these types of background information to the roundtable panel, the PC asked Barry Shane (6) of the University of California, Berkeley, to discuss the history of folate measurement and assessment, and Ralph Carmel (7) of Weill Medical College of Cornell University to do the same for vitamin B-12 (Table 2).

Finally, part of the roundtable’s charge was to consider which biomarkers and associated measurement procedures to include in future NHANES. Public health concerns related to folate status are currently changing from inadequacy to overages and possible adverse effects. The PC therefore recommended that the roundtable review the potential usefulness of the inclusion of measures of folate vitamins in future NHANES and consider available measurement procedures for their possible use. Because Jacob Selhub’s group at Tufts University had measured folate vitamins from surplus sera from NHANES, the PC asked Selhub to describe the measurement procedures his group used for that special research project (Table 2) (10). After it learned that Pfeiffer’s group was evaluating a liquid chromatography–tandem mass spectrometry (LC-MS/MS) procedure for measurement of folate vitamins, the PC asked Pfeiffer to describe the measurement procedure she used (2). To help the roundtable understand the potential public health ramifications of the measurement of folate vitamins in future NHANES, Regan Bailey of ODS described her population-based results with the use of the NHANES 2001–2002 serum folic acid (also referred to as unmetabolized folic acid) data that Tufts University had generated in a special surplus sera research project (Table 1) (11).

The roundtable also considered potential issues for the assessment of vitamin B-12 status in the future. Because of increased interest in the measurement of holotranscobalamin in clinical and research settings, the PC asked the roundtable to review the possible inclusion of this measurement procedure in future NHANES. Ebba Nexo of Aarhus University Hospital in Denmark presented background information on the development and usefulness of this measure for population-based surveys (Table 2) (8). Carmel also covered this topic briefly, from a somewhat different perspective, in his background information (7). To help the roundtable evaluate the usefulness of vitamin B-12 status data to NHANES users, Bailey used data from NHANES 1999–2004 to evaluate the population-based results of the combination of serum vitamin B-12 and MMA to assess vitamin B-12 status (3).

KEY POINTS FROM THE ROUNDTABLE DIALOGUE

The ODS and the Division of Health and Nutrition Examination Surveys charged the roundtable with identification of the key scientific issues and discussion of the pros and cons of these issues but not with the making of recommendations. With limited time for dialogue, the roundtable focused its attention on the issues that members identified as most critical for the folate- and vitamin B-12–related biomarkers in NHANES (2, 3). Therefore, the roundtable’s dialogue did not cover all possible topics and the group did not necessarily take time to resolve differences of opinion in the background presentations. In some cases, the roundtable achieved general agreement on an issue; in other cases, group members expressed different perspectives, which the dialogue summaries capture.

For both folate and vitamin B-12, the issues that had plagued past expert panels also caught the attention of the 2010 roundtable: 1) the interactions of measurement procedures and cutoffs of adequacy, 2) the effect of changes in measurement procedures on time-trend analysis, 3) the public health importance of various measures, and 4) the use of evolving science to improve NHANES measurement procedures and interpretability. In addition, like the 2009 roundtable (4), the 2010 roundtable acknowledged the urgent need to ensure accurate NHANES biomarker measurements through the application of traceability approaches with the use of NIST and other external reference materials.

Folate-related background issues

The roundtable’s discussions focused on measurement and biomarker issues (Table 3). In general, the roundtable agreed that the MA is the traditional gold standard against which to evaluate other measurement procedures (2, 6). It is also the basis for currently used cutoffs of adequacy. On the basis of published literature that shows substantially lower results with the use of radioimmunoassay procedures compared with MA, the roundtable was not surprised by the marked differences between the Bio-Rad Quantaphase II (Bio-Rad Laboratories, Hercules, CA) and the MA procedures that Pfeiffer described. The roundtable agreed that an adjustment equation based on a crossover study was necessary for time-trend evaluations.

The roundtable’s review of the NCEH data that showed close agreement between the LC-MS/MS and MA measurement
procedures for serum folates formed the basis for the group’s suggestion to base serum folate measures in future NHANES on the LC-MS/MS procedure, which includes measurement of serum vitamers. When the roundtable learned of the success of the NCEH’s LC-MS/MS procedure for the measurement of serum total folates and some folate vitamers, it did not find it necessary to also consider other measurement procedures, such as the affinity HPLC measurement procedure that Selhub described (10). However, Shane noted the potential usefulness of the HPLC method for laboratories that lack LC-MS/MS facilities (6). The situation for RBC folates was less clear. LC-MS/MS results differ from MA results by ~25%. The roundtable therefore agreed that the use of the LC-MS/MS procedure for measurement of RBC folates requires further research before future NHANES can measure RBC folates by LC-MS/MS procedures.

The roundtable spent considerable time in a discussion about whether future NHANES need to measure both serum and RBC folate and if NHANES only measures one of these, which one is best. After considerable discussion, the group decided that either serum or RBC folate measures would be useful for future NHANES.

Vitamin B-12–related background issues

Because it was generally satisfied with the NCEH’s handling of measurement procedure issues for vitamin B-12–related biomarkers, the roundtable turned its attention to reasons to reinstate vitamin B-12 biomarkers in future NHANES and, if NHANES does measure vitamin B-12 biomarkers in the future, which biomarkers the surveys should measure (Table 4) (3). No NHANES since NHANES 2006 has measured any of these biomarkers. The roundtable agreed with Carmel about the lack of confirmation that subclinical vitamin B-12 deficiency (also referred to as subclinical cobalamin deficiency or SCCD), the predominant form of vitamin B-12 inadequacy in the US population, presents a public health concern (7). However, roundtable members also noted concerns that observational studies have raised about possible adverse effects, and emphasized the need for further investigation of these effects, particularly in clinical trials. The roundtable identified several factors that may justify the reinstatement of vitamin B-12 measures in future NHANES.

The roundtable generally agreed that NHANES would preferably measure ≥2 vitamin B-12 biomarkers because of problems with sensitivity and specificity of any single biomarker (3). If NHANES includes 2 measures, it would be useful if one was a measure of circulating concentrations of vitamin B-12 (ie, serum vitamin B-12 or holotranscobalamin) and one a functional measure of vitamin B-12 inadequacy (ie, plasma MMA or tHcy). The roundtable agreed that, although holotranscobalamin holds promise for a more meaningful measure of circulating vitamin B-12 than does serum vitamin B-12, sufficient experience is not available for the measurement and use of holotranscobalamin to include it as the sole measure of circulating concentrations of vitamin B-12. MMA was preferable to tHcy because of its specificity for vitamin B-12 inadequacies.

In summary, the 2010 roundtable agreed that a move toward the development and use of traceability approaches is the best way to deal with accuracy and comparability problems in NHANES biomarker measurements. An urgent need exists for trials that relate accurately measured nutrient biomarker data to clinical outcomes to identify cutoffs of nutrient adequacy. Either serum or RBC folate would be useful for future NHANES. The use of the LC-MS/MS procedure for measurement of RBC folates requires further research.
before future NHANES can use this approach to measure RBC folates, but this procedure can now be used to measure serum folate. If NHANES reinstates vitamin B-12 biomarker measurement, the surveys should include at least one measure of circulating concentrations of vitamin B-12 and one functional measure of vitamin B-12 inadequacy.

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