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Applying inappropriate cutoffs leads to misinterpretation of folate status in the US population[1–3]

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ABSTRACT

Background: Folate cutoffs for risk of deficiency compared with possible deficiency were originally derived differently (experimental compared with epidemiologic data), and their interpretations are different. The matching of cutoffs derived from one assay with population-based data derived from another assay requires caution.

Objective: We assessed the extent of folate-status misinterpretation with the use of inappropriate cutoffs.

Design: In the cross-sectional NHANES, serum and red blood cell (RBC) folate were first measured with the use of a radioprotein-binding assay (RPBA) (1988–2006) and, afterwards, with the use of a microbiologic assay (2007–2010). We compared prevalence estimates for assay-matched cutoffs (e.g., with the use of an RPBA cutoff with RPBA data) and assay-mismatched cutoffs (e.g., with the use of microbiologic assay cutoff with RPBA data) for risk of deficiency on the basis of megaloblastic anemia as a hematologic indicator in persons ≥4 y of age (e.g., serum folate concentration <7 nmol/L and RBC folate concentration <305 nmol/L derived with the use of a microbiologic assay), possible deficiency on the basis of rising homocysteine as a metabolic indicator in persons ≥4 y of age (e.g., serum folate concentration <10 nmol/L and RBC folate concentration <340 nmol/L derived with the use of an RPBA), and insufficiency on the basis of elevated risk of neural tube defects in women 12–49 y old (e.g., RBC folate concentration <906 nmol/L derived with the use of a microbiologic assay).

Results: Pre–folic acid fortification (1988–1994), risks of deficiency for assay-matched compared with assay-mismatched cutoffs were 5.6% compared with 16% (serum folate), respectively, and 7.4% compared with 28% (RBC folate), respectively; risks declined postfortification (1999–2006) to <1% compared with <1% (serum folate), respectively, and to <1% compared with 2.5% (RBC folate), respectively. Prefortification (1988–1994), risks of possible deficiency for assay-matched compared with assay-mismatched cutoffs were 35% compared with 56% (serum folate), respectively, and 37% compared with 84% (RBC folate), respectively; risks declined postfortification (1999–2006) to 1.9% compared with 7.0% (serum folate), respectively, and to 4.8% compared with 53% (RBC folate), respectively. Postfortification (2007–2010), risks of insufficiency were 3% (assay matched) compared with 39% (assay mismatched), respectively.

Conclusions: The application of assay-mismatched cutoffs leads to a misinterpretation of folate status. This confusion likely applies to clinical assays because no comparability data are available, to our knowledge. *Am J Clin Nutr* 2016;104:1607–15.

Keywords: deficiency, insufficiency, microbiologic assay, NHANES, radioprotein-binding assay

INTRODUCTION

Low folate status has been associated with increased risks of adverse health outcomes from megaloblastic anemia and hyperhomocysteinemia to elevated risk of a neural tube birth-defect–affected pregnancy (1). The use of appropriate cutoffs is essential to correctly assess folate status (2). The WHO summarized historical information on serum and red blood cell (RBC)[10] folate concentrations for the assessment of folate status in populations (3). A recent folate review stated that the inconsistent use of cutoffs over time has led to scientific confusion (4). Earlier reports concluded that, because of large assay differences, method-specific reference ranges should be used (5, 6). Large assay differences are still seen today with proficiency testing data for clinical assays. The 2015 College of American Pathologists Ligand Survey showed large within-platform variability (~7–30% and ~10–50% for

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[2] Findings and conclusions in this manuscript are those of the authors and do not necessarily represent the official views or positions of the CDC/Agency for Toxic Substances and Disease Registry, the NIH, or the Department of Health and Human Services. In addition, the views expressed in this article do not necessarily represent the decisions, policies, or views of the WHO.

[3] Supplemental Figure 1, Supplemental Text 1, and Supplemental Tables 1–8 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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[10] Abbreviations used: Hcy, plasma homocysteine; IOM, Institute of Medicine; MBA-1, microbiologic assay with wild-type microorganism and folic acid calibrator; MBA-2, microbiologic assay with chloramphenicol-resistant strain and folic acid calibrator; MBA-3, microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator; NTD, neural tube defect; RBC, red blood cell; RPBA, radioprotein-binding assay.

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serum and RBC folate, respectively) and even larger across-platform variability (~1.5- to 6-fold and ~8- to 40-fold differences for serum and RBC folate, respectively) (GL Horowitz, DN Alter, Chemistry Resource Committee, personal communication, 2015). We know how folate assays used in the NHANES compare (7) and can calculate assay-adjusted cutoffs; however, this type of information is lacking for commonly used clinical assays, resulting in an inability to appropriately compare study data produced with different clinical assays or interpret patient data.

The 1998 Institute of Medicine (IOM) Dietary Reference Intakes report (1) and WHO guidelines (3, 8, 9) are primary sources for folate cutoffs (**Table 1**). Folate-depletion experiments conducted with the use of a microbiologic assay [microbiologic assay with wild-type microorganism and folic acid calibrator (MBA-1) (14)] have defined stages of deficiency with megaloblastic anemia being the final stage of deficiency on the basis of a hematologic indicator. Serum folate concentrations <7 nmol/L indicated a negative balance (14). RBC folate concentrations <363, <272, and <227 nmol/L were markers of the onset of depletion, the beginning of deficient erythropoiesis, and megaloblastic anemia, respectively (14). An RBC folate cutoff <305 nmol/L, which indicated the appearance of hypersegmented neutrophils, was commonly used (1, 5). A 2005 WHO Technical Consultation recommended cutoffs for possible deficiency on the basis of rising plasma homocysteine (Hcy) as a metabolic indicator (serum folate concentration <10 nmol/L and RBC folate concentration <340 nmol/L) (15, 16). These cutoffs were derived from cross-sectional NHANES 1988–1994 data with the use of a Bio-Rad radioprotein-binding assay (RPBA) (Bio-Rad Laboratories). In 2015, the WHO recommended a population cutoff for folate insufficiency in women of reproductive age on the basis of elevated risk of neural tube defects (NTDs) [RBC folate concentration <906 nmol/L (9)] that was derived from epidemiologic data produced by a microbiologic assay [microbiologic assay with chloramphenicol-resistant strain and folic acid calibrator (MBA-2) (17)].

This study assessed commonly used folate cutoffs that were derived experimentally or from epidemiologic data and cutoff adjustments to obtain assay matching. We applied assay-matched cutoffs (e.g., RPBA cutoffs with RPBA data) and assay-mismatched cutoffs (e.g., MBA-1 cutoffs with RPBA data) for different levels of folate status (risk of deficiency on the basis of megaloblastic anemia, possible deficiency on the basis of rising Hcy, and insufficiency on the basis of elevated NTD risk) to serum and RBC folate data from persons ≥4 y old who were participating in the NHANES 1988–2010 and compared the resulting prevalence estimates and the extent of misinterpretation of folate status.

METHODS

Participants and study design

The NHANES, which is conducted by the National Center for Health Statistics at the CDC, collects nationally representative cross-sectional data on the health and nutritional status of the civilian, noninstitutionalized US population with the use of a stratified, multistage, probability sample design. Survey participants were first interviewed in their homes to collect information on demographic characteristics, health-related issues, and dietary

TABLE 1
Commonly used cutoffs to assess folate status and their assay-adjusted equivalents[1]

Type of deficiency matrix	Published cutoff, nmol/L (reference)[2]	Assay for cutoff[3]	Type of data	Interpretation	Adjusted cutoff, nmol/L (reference)[4]	Target assay[5]
Deficiency on the basis of hematologic indicator						
Serum	<7 (1, 3)	MBA-1	Experimental	Negative folate balance	<5 (10)	RPBA
RBC	<305 (1)	MBA-1	Experimental	Appearance of hypersegmented neutrophils[6]	<215 (11)	RPBA
RBC	<227 (3)	MBA-1	Experimental	Megaloblastic anemia	<156 (10)	RPBA
Possible deficiency on the basis of metabolic indicator						
Serum	<10 (3)	RPBA	Epidemiologic	Rising homocysteine	<14 (7)[7]	MBA-3
RBC	<340 (3)	RPBA	Epidemiologic	Rising homocysteine	<624 (7)[7]	MBA-3
Insufficiency on the basis of elevated neural tube–defect risk						
RBC	<906 (9)	MBA-2	Epidemiologic	Limited to WRA at the population level	<748 (12, 13)	MBA-3

[1] All RPBAs shown in the table are BioRad RPBAs (Bio-Rad Laboratories). MBA-1, microbiologic assay with wild-type microorganism and folic acid calibrator; MBA-2, microbiologic assay with chloramphenicol-resistant strain and folic acid calibrator; MBA-3, microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator; RBC, red blood cell; RPBA, radioprotein-binding assay; WRA, women of reproductive age.

[2] Cutoffs expressed in conventional units are as follows: deficiency: 3 ng/mL (serum), 140 ng/mL (RBC), and 100 ng/mL (RBC); possible deficiency: 4 ng/mL (serum) and 151 ng/mL (RBC); and insufficiency: 400 ng/mL (RBC).

[3] Assay used to generate folate results on which the cutoff was based.

[4] Cutoffs expressed in SI units were rounded to integers to avoid giving inappropriate implied precision as follows: deficiency: 4.5 rounded to 5 nmol/L (serum), 215.3 rounded to 215 nmol/L (RBC), and 156.4 rounded to 156 nmol/L (RBC); possible deficiency: 13.7 rounded to 14 nmol/L (serum) and 623.6 rounded to 624 nmol/L (RBC); and insufficiency: 747.8 rounded to 748 nmol/L (RBC). Cutoffs expressed in conventional units are as follows: deficiency: 2 ng/mL (serum), 95 ng/mL (RBC), and 69 ng/mL (RBC); possible deficiency: 6 ng/mL (serum) and 275 ng/mL (RBC); and insufficiency: 330 ng/mL (RBC).

[5] Assay to which adjusted cutoff was applied.

[6] Lack of megaloblastic changes in subjects with RBC folate concentrations >140 ng/mL.

[7] Reference provides regression equations to calculate adjusted cutoffs.

supplement use. Participants underwent a physical examination and a blood draw in a Mobile Examination Center ~1–3 wk after the household interview. All NHANES participants provided written informed consent, and all procedures were approved by the National Center for Health Statistics Research Ethics Review Board.

Biomarker measurement

The BioRad Quantaphase I RPBA (Bio-Rad Laboratories) was used in 1988–1991, and the Quantaphase II RPBA (Bio-Rad Laboratories) was used in 1991–2006, to measure folate in serum and whole-blood hemolysate samples for RBC folate determination (**Supplemental Table 1**). Quantaphase I data were assay adjusted before their public release to account for assay differences between the Quantaphase I and II (10). The CDC microbiologic assay [microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator (MBA-3)], was used in 2007–2010 to measure folate in serum and whole-blood hemolysate samples (18).

Study variables

This study used data from persons aged ≥4 y who were participating in the NHANES 1988–2010. For sample sizes, see **Supplemental Table 2**. We used the demographic variables age, sex, and race-ethnicity and categorized them as follows: age (4–11, 12–19, 20–39, 40–59, and ≥60 y), sex (males and females), and race-ethnicity (Mexican American, non-Hispanic black, and non-Hispanic white; other race-ethnicity groups were included in the overall estimates). Women of reproductive age (12–49 y) were considered separately to assess elevated NTD risk. The use of any dietary supplement was ascertained from self-reported use over the past 30 d (yes and no; with the use of data from the dietary supplement questionnaire).

Statistics

Statistical analyses were performed with the use of SAS software (version 9; SAS Institute Inc.) and SUDAAN software (version 9.2; RTI). We applied no a priori exclusion criteria to our data analysis and used pairwise deletion for missing values in a particular bivariate analysis. Mobile Examination Center statistical weights were used to account for a differential nonresponse or noncoverage and to adjust for the oversampling of some groups. We assessed the proportion of individuals at different levels of folate status with the use of 3 approaches. First, we calculated the weighted prevalence of low serum and RBC folate concentrations at various assay-matched cutoffs (e.g., BioRad RPBA cutoff with BioRad RPBA data) with the use of the original data as measured by the laboratory. Second, we applied various assay-mismatched cutoffs (e.g., MBA-1 cutoff with BioRad RPBA data) to the original data as measured by the laboratory. Third, we adjusted the original data and applied assay-matched cutoffs to calculate the weighted prevalence. We did not assess the fourth possibility of applying assay-mismatched cutoffs to adjusted data. To facilitate the data review, we compiled the cutoffs that were used for each of these 3 approaches (**Table 2**) and summarized information on the assays underlying the cutoffs and the NHANES data (**Table 3**) in the same format as we later present the data. We used MBA-1 cutoffs for risk of deficiency on the basis of megaloblastic anemia with MBA-3 data and considered this scenario to be likely assay matched. We know of no regression equation that links the MBA-1 and MBA-3 assays, and we consider these 2 assays to produce

TABLE 2
Assay-matched and assay-mismatched cutoffs for risks of folate deficiency, possible deficiency, or insufficiency applied to original or adjusted data from participants aged ≥4 y in the prefortification (1988–1994) and postfortification (1999–2010) NHANES[1]

	Serum folate, nmol/L			RBC folate, nmol/L		
Type of deficiency and cutoff, data	1988–1994	1999–2006	2007–2010	1988–1994	1999–2006	2007–2010
Deficiency on the basis of hematologic indicator (megaloblastic anemia)						
Assay matched, original	<5[2]	<5[2]	<7	<215[2]	<215[2]	<305
Assay mismatched, original	<7	<7	<5[2]	<305	<305	<215[2]
Assay matched, adjusted	<7	<7	ND	<305	<305	ND
Possible deficiency on the basis of metabolic indicator (rising homocysteine)						
Assay matched, original	<10	<10	<14[2]	<340	<340	<624[2]
Assay mismatched, original	<14[2]	<14[2]	<10	<624[2]	<624[2]	<340
Assay matched, adjusted	<14[2]	<14[2]	ND	<624[2]	<624[2]	ND
Insufficiency on the basis of elevated neural tube defect risk[3]						
Assay matched, original	NA	NA	NA	ND	ND	<748[2]
Assay mismatched, original	NA	NA	NA	<906	<906	<906
Assay matched, adjusted	NA	NA	NA	<748[2]	<748[2]	ND

[1] Original data for 1988–2006 were generated with the BioRad radioprotein-binding assay (Bio-Rad Laboratories); original data for 2007–2010 were generated with the CDC MBA-3 (MBA-3). Data marked as ND for 1988–1994 and 1999–2006 were due to no BioRad cutoff being available for folate insufficiency; data marked as ND for 2007–2010 were due to MBA-3 data that were not adjusted. MBA-3, microbiologic assay with chloramphenicolresistant strain and 5-methyltetrahydrofolate calibrator; NA, not applicable; ND, not determined; RBC, red blood cell.

[2] Cutoff was adjusted.

[3] Limited to women 12–49 y of age.

TABLE 3
Assay underlying each cutoff and each NHANES data set to determine risks of folate deficiency, possible deficiency, or insufficiency for participants aged ≥4 y in the prefortification (1988–1994) and postfortification (1999–2010) NHANES[1]

	Assay for cutoff/assay for NHANES data		
Type of deficiency and cutoff, data	1988–1994	1999–2006	2007–2010
Deficiency on the basis of hematologic indicator (megaloblastic anemia)			
Assay matched, original	$RPBA_{Adj}$/RPBA	$RPBA_{Adj}$/RPBA	MBA-1/MBA-3
Assay mismatched, original	MBA-1/RPBA	MBA-1/RPBA	$RPBA_{Adj}$/MBA-3
Assay matched, adjusted	MBA-1/$MBA\text{-}3_{Adj}$	MBA-1/$MBA\text{-}3_{Adj}$	ND
Possible deficiency on the basis of metabolic indicator (rising homocysteine)			
Assay matched, original	RPBA/RPBA	RPBA/RPBA	$MBA\text{-}3_{Adj}$/MBA-3
Assay mismatched, original	$MBA\text{-}3_{Adj}$/RPBA	$MBA\text{-}3_{Adj}$/RPBA	RPBA/MBA-3
Assay matched, adjusted	$MBA\text{-}3_{Adj}$/$MBA\text{-}3_{Adj}$	$MBA\text{-}3_{Adj}$/$MBA\text{-}3_{Adj}$	ND
Insufficiency on the basis of elevated neural tube defects risk[2]			
Assay matched, original	ND	ND	$MBA\text{-}3_{Adj}$/MBA-3
Assay mismatched, original	MBA-2/RPBA	MBA-2/RPBA	MBA-2/MBA-3
Assay matched, adjusted	$MBA\text{-}3_{Adj}$/$MBA\text{-}3_{Adj}$	$MBA\text{-}3_{Adj}$/$MBA\text{-}3_{Adj}$	ND

[1] Subscript Adj indicates that the cutoff was adjusted or the NHANES data were adjusted, whereas no subscript indicates that the published cutoff was used or the original NHANES data were used. Data marked as ND for 1988–1994 and 1999–2006 were due to no BioRad cutoff (Bio-Rad Laboratories) being available for folate insufficiency; data marked as ND for 2007–2010 were due to MBA-3 data that were not adjusted. MBA-1, microbiologic assay with wild-type microorganism and folic acid calibrator; MBA-2, microbiologic assay with chloramphenicol-resistant strain and folic acid calibrator; MBA-3, microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator; ND, not determined; RPBA, radioprotein-binding assay.

[2] Limited to women 12–49 y of age.

similar concentrations (see Discussion). We forward adjusted the BioRad RPBA data from the NHANES 1988–1994 and 1999–2006 to MBA-3 units (fractional polynomial regression for serum folate and linear regression for whole-blood folate) (Supplemental Table 1) with the use of previously published regression equations (7) to have data for the full 1988–2010 time period in the same MBA-3 units. We also used these regression equations to calculate assay-adjusted MBA-3 cutoffs for risk of possible deficiency on the basis of rising Hcy. When we adjusted the BioRad RPBA data, we excluded serum folate data from 2 participants in 1988–1994 because their unadjusted concentrations were <1 nmol/L, and the adjustment formula required logarithmic transformation (which produces a negative number) and then the calculation of the square root. We also excluded RBC folate data for a small fraction (1–2%) of participants in 1988–1994 (558 of 23,404) and 1999–2006 (469 of 31,278) because the RBC folate–adjustment formula required serum folate, RBC folate, and hematocrit, and for these participants, one of these tests was missing. To provide a visual representation of where in the distribution the cutoffs lie, we generated cumulative frequency-distribution curves of original and adjusted serum and RBC folate concentrations for the 3 time periods (1988–1994, 1999–2006, and 2007–2010) (**Supplemental Figure 1**). Regression equations that show the relations between 3 microbiologic assays that are currently used [MBA-2 (17), MBA-3 (18), and a variation of MBA-1 calibrated with 5-formyltetrahydrofolate (19)], although beyond the scope of this paper, are often required by researchers to interpret published data (**Supplemental Text 1**).

RESULTS

Cutoff adjustments to achieve assay matching

Folate cutoffs published by the IOM and WHO have to be assay matched when used with NHANES data to assess long-term temporal trends (Table 1). Cutoffs for risk of deficiency on the basis of megaloblastic anemia as a hematologic indicator (serum folate concentration <7 nmol/L and RBC folate concentration <305 nmol/L) were mainly derived from MBA-1 data and could not be used directly with the BioRad RPBA data from the NHANES 1988–1994 and 1999–2006. An earlier report (10) published a regression equation to convert BioRad RPBA Quantaphase I data to Quantaphase II data on the basis of results from nearly 1800 serum samples and showed that the Quantaphase II assay measured ~35% lower. Because the Quantaphase I assay was originally calibrated to the MBA-1, the investigators used the same regression equation to calculate adjusted cutoffs for Quantaphase II data (serum folate concentration <5 nmol/L and RBC folate concentration <215 nmol/L) (10, 11).

WHO cutoffs for risk of possible deficiency on the basis of rising Hcy as a metabolic indicator (serum folate concentration <10 nmol/L and RBC folate concentration <340 nmol/L) were derived from prefortification BioRad RPBA data (3, 15, 16). These cutoffs can be used directly with data from the NHANES 1988–1994 and 1999–2006; however, adjusted cutoffs have to be used with MBA-3 data from the NHANES 2007–2010 (3). The MBA-3 measured ~40% higher (serum) and 80% higher (RBC) than the BioRad RPBA did (7), and the adjusted MBA-3 cutoffs were <14 nmol/L for serum folate concentrations and <624 nmol/L for RBC folate concentrations.

Finally, the WHO cutoff for insufficiency on the basis of elevated NTD risk in women of reproductive age (RBC folate concentration <906 nmol/L) was derived from the MBA-2 data [assay calibrated with folic acid (17)] and could not be used directly with NHANES data. The MBA-3 assay used in the NHANES 2007–2010 (18) was calibrated with 5-methyltetrahydrofolate, which, compared with calibration with folic acid, produced a higher microorganism growth response (i.e., a higher calibration curve) and ~25% lower folate concentrations in patient samples (12). The adjusted MBA-3 cutoff was <748 nmol/L for RBC folate concentrations (13).

Risk of folate deficiency on the basis of hematologic indicator with the use of original data

Risks of deficiency on the basis of megaloblastic anemia for assay-matched cutoffs (e.g., BioRad RPBA cutoff with BioRad RPBA data) were 5.6% (serum folate) and 7.4% (RBC folate), respectively, during pre–folic acid fortification (1988–1994); risk of deficiency declined postfortification (1999–2006 and 2007–2010) to <1% (serum and RBC folate) (**Table 4**). The same prefortification to postfortification pattern, but with a slightly different magnitude, was observed for different age and race-ethnicity groups, males and females, and supplement users and nonusers (**Table 5**). When we used assay-mismatched cutoffs (e.g., MBA-1 cutoff with BioRad RPBA data), risks of folate deficiency on the basis of megaloblastic anemia were overestimated during the prefortification period (16% for serum folate and 28% for RBC folate) and slightly overestimated for RBC folate during the 1999–2006 postfortification period (2.5%) (Table 4). Estimates by population subgroup varied to the greatest extent by age group in the prefortification period as follows: 1.3% for 4–11-y-olds compared with 23% for 20–39-y-olds for serum folate and 12% for 4–11-y-olds compared with 39% for 12–19-y-olds for RBC folate (**Supplemental Table 3**).

Risk of possible folate deficiency on the basis of metabolic indicator with the use of original data

When we used assay-matched cutoffs, risks of possible deficiency on the basis of rising Hcy declined from 35% (serum folate) and 37% (RBC folate) prefortification to 1.9% (1999–2006) and 4.2% (2007–2010) for serum folate and to 4.8% (1999–2006) and 8.6% (2007–2010) for RBC folate postfortification (Table 4). As noted with risk of deficiency on the basis of megaloblastic anemia, we observed the same prefortification to postfortification patterns for different demographic groups and supplement users and nonusers (**Table 6**). When we used assay-mismatched cutoffs, risks of possible folate deficiency on the basis of rising Hcy were overestimated during 1988–1994 (56% for serum folate and 84% for RBC folate) and 1999–2006 (7.0% for serum folate and 53% for RBC folate) and were underestimated during 2007–2010 (<1% for serum and RBC folate) (Table 4). Estimates by population subgroup varied to the greatest extent by age group in the early postfortification period (1999–2006) as follows: 0.3% for 4–11-y-olds compared with 10% for 20–39-y-olds for serum folate and 32% for ≥60-y-olds compared with 72% for 12–19-y-olds for RBC folate (**Supplemental Table 4**).

TABLE 4
Prevalence for risks of folate deficiency, possible deficiency, or insufficiency for the total population with the use of assay-matched or assay-mismatched cutoffs and original or assay-adjusted data from participants aged ≥4 y in the prefortification (1988–1994) and postfortification (1999–2010) NHANES[1]

	Serum folate			RBC folate		
Type of deficiency and cutoff, data	1988–1994	1999–2006	2007–2010	1988–1994	1999–2006	2007–2010
Deficiency on the basis of hematologic indicator (megaloblastic anemia)						
Assay matched, original	5.6 (4.8, 6.5)[2]	0.1 (0.0, 0.1)[2,*]	0.1 (0.1, 0.2)	7.4 (6.5, 8.4)[2]	0.2 (0.2, 0.3)[2]	0.1 (0.1, 0.3)*
Assay mismatched, original	16 (15, 18)	0.4 (0.3, 0.5)	<1[2,†]	28 (26, 30)	2.5 (2.2, 2.8)	<1[2,†]
Assay matched, adjusted	16 (14, 17)	0.3 (0.3, 0.5)	ND	2.0 (1.6, 2.4)	0.1 (0.0, 0.1)	ND
Possible deficiency on the basis of metabolic indicator (rising homocysteine)						
Assay matched, original	35 (33, 38)	1.9 (1.7, 2.1)	4.2 (3.7, 4.8)[2]	37 (35, 39)	4.8 (4.3, 5.3)	8.6 (7.7, 9.5)[2]
Assay mismatched, original	56 (53, 58)[2]	7.0 (6.4, 7.5)[2]	0.7 (0.5, 1.0)	84 (82, 85)[2]	53 (51, 55)[2]	0.2 (0.1, 0.3)
Assay matched, adjusted	37 (35, 40)[2]	2.2 (1.9, 2.4)[2]	ND	37 (35, 39)[2]	4.7 (4.2, 5.2)[2]	ND
Insufficiency on the basis of elevated neural tube defects risk[3]						
Assay matched, original	NA	NA	NA	ND	ND	23 (21, 25)[2]
Assay mismatched, original	NA	NA	NA	97 (96, 98)	89 (87, 90)	39 (36, 41)
Assay matched, adjusted	NA	NA	NA	59 (56, 63)[2]	15 (14, 17)[2]	ND

[1] All values are percentages (95% CIs). For cutoffs used, see Table 2; for sample sizes, see Supplemental Table 2. The other race-ethnicity group (persons with multiethnic background) was included in these total estimates. Original data for 1988–2006 were generated with a BioRad radioprotein-binding assay (Bio-Rad Laboratories); original data for 2007–2010 were generated with the CDC microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator. Data marked as ND for 1988–1994 and 1999–2006 were due to no BioRad cutoff being available for folate insufficiency; data marked as ND for 2007–2010 were due to microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator that were not adjusted. *Relative SE ≥30% and <40%. †Estimate was suppressed; relative SE ≥40%. NA, not applicable; ND, not determined; RBC, red blood cell.

[2] Cutoff was adjusted.

[3] Limited to women 12–49 y of age.

TABLE 5
Prevalence for risk of folate deficiency by population subgroup with the use of assay-matched cutoffs and original data from participants aged ≥4 y in the prefortification (1988–1994) and postfortification (1999–2010) NHANES[1]

	Serum folate cutoff, nmol/L (and time period)			RBC[2] folate cutoff, nmol/L (and time period)		
Population group	<5[3] (1988–1994)	<5[3] (1999–2006)	<7[4] (2007–2010)	<215[3] (1988–1994)	<215[3] (1999–2006)	<305[4] (2007–2010)
Age group, y						
4–11	<1†	<1†	<1†	1.5 (1.1, 2.2)	<1†	<1†
12–19	4.9 (3.8, 6.5)	<1†	<1†	12 (9.5, 14)	0.2 (0.1, 0.3)	<1†
20–39	8.7 (7.2, 10)	<1†	<1†	8.9 (7.6, 10)	0.2 (0.1, 0.4)	<1†
40–59	5.8 (4.9, 6.9)	<1†	<1†	8.0 (6.7, 9.4)	0.4 (0.2, 0.7)	<1†
≥60	2.8 (2.2, 3.8)	<1†	<1†	4.5 (3.8, 5.4)	0.2 (0.1, 0.3)	<1†
Sex						
Male	5.8 (4.8, 7.1)	<1†	0.1 (0.0, 0.2)*	6.6 (5.5, 7.8)	0.2 (0.2, 0.4)	<1†
Female	5.3 (4.5, 6.3)	<1†	0.1 (0.0, 0.2)*	8.2 (7.2, 9.4)	0.2 (0.1, 0.4)	0.2 (0.1, 0.4)*
Race-ethnicity						
Mexican American	5.8 (4.9, 6.7)	<1†	<1†	7.0 (5.5, 8.7)	0.3 (0.1, 0.5)*	<1†
Non-Hispanic black	7.7 (6.7, 8.9)	<1†	0.3 (0.1, 0.6)*	18 (16, 20)	0.9 (0.6, 1.2)	<1†
Non-Hispanic white	5.5 (4.5, 6.6)	<1†	0.1 (0.0, 0.2)*	6.0 (5.0, 7.2)	<1†	<1†
Supplement use						
Yes	7.0 (5.8, 8.3)	<1†	<1†	6.5 (5.1, 8.3)	0.2 (0.1, 0.3)	<1†
No	4.9 (4.0, 6.0)	0.1 (0.1, 0.2)*	0.2 (0.1, 0.3)*	7.8 (6.7, 9.1)	0.3 (0.2, 0.4)	0.2 (0.1, 0.4)*

[1] All values are percentages (95% CIs). Data were determined on the basis of megaloblastic anemia as a hematologic indicator. Data for 1988–2006 were generated with a BioRad radioprotein-binding assay (Bio-Rad Laboratories); data for 2007–2010 were generated with the CDC microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator. *Relative SE ≥30% and <40%. †Estimate was suppressed; relative SE ≥40%.
[2] RBC, red blood cell.
[3] Microbiologic assay with wild-type microorganism and folic acid calibrator cutoffs of 7 nmol/L (serum folate) and 305 nmol/L (RBC folate) were adjusted to BioRad radioprotein-binding assay units (10).
[4] Microbiologic assay with wild-type microorganism and folic acid calibrator cutoff was used as is.

Risk of folate insufficiency on the basis of elevated NTD risk with the use of original data

During 2007–2010, elevated NTD risks in women of reproductive age (12–49 y) were 23% compared with 39% when we used an assay-matched cutoff compared with an assay-mismatched cutoff, respectively (Table 4). In both cases, estimates varied by race-ethnicity and supplement use status, but prefortification to postfortification patterns were consistent with our observation in the total population (**Supplemental Table 5** and **Table 7**).

Risk of folate deficiency and possible deficiency with the use of assay-adjusted data

When we used adjusted data (i.e., applied regression equations to BioRad RPBA data to adjust them to MBA-3 units) and applied MBA-1 cutoffs (Table 4), we observed different estimates for risk of deficiency on the basis of megaloblastic anemia during the prefortification period than were obtained with the use of original BioRad RPBA data and adjusting the MBA-1 cutoffs to BioRad units (16% compared with 5.6% for serum folate and 2.0% compared with 7.4% for RBC folate). During the postfortification period (1999–2006), we observed no difference in prevalence between these 2 approaches (<1% for serum and RBC folate). Similarly, the estimates for risk of possible deficiency on the basis of rising Hcy were comparable between the 2 approaches both for the prefortification period (1988–1994; 37% compared with 35% for serum folate and 37% compared with 37% for RBC folate) and postfortification period (1999–2006; 2.2% compared with 1.9% for serum folate and 4.7% compared with 4.8% for RBC folate). Estimates by population subgroup varied to the greatest extent by age group in the prefortification period as follows: 1.1% for 4–11-y-olds compared with 22% for 20–39-y-olds for serum folate risk of deficiency on the basis of megaloblastic anemia (**Supplemental Table 6**) and 8.7% for 4–11-y-olds compared with 48% for 20–39-y-olds for serum folate risk of possible deficiency on the basis of rising Hcy (**Supplemental Table 7**). Risk of folate insufficiency on the basis of elevated NTD risk varied by race-ethnicity both pre- and postfortification, but by supplement use only postfortification (**Supplemental Table 8**).

DISCUSSION

This article provides information on commonly used folate cutoffs that were derived from experimental or epidemiologic data and on adjustments to achieve assay matching, thereby making it an important tool for researchers and public health program and policy officials to evaluate population folate status. Our data also provide correct prevalence estimates for 3 levels of folate status by population subgroup (Tables 5–7). The use of assay-mismatched cutoffs led to a misinterpretation of folate status. The extent of misinterpretation depended largely on the presence of low folate concentrations in the population and, therefore, was different in the prefortification period than in the postfortification period and in different population subgroups. It appeared that subgroups with a low prevalence of low folate concentrations (e.g., children aged 4–11 y) were more sensitive to misinterpretation. Although this article does not address the extent of misinterpretation at the individual level in a clinical setting or with any laboratory-developed assay, there is clearly a need for more research in this area.

TABLE 6
Prevalence for risk of possible folate deficiency by population subgroup with the use of assay-matched cutoffs and original data from participants aged ≥4 y in the prefortification (1988–1994) and postfortification (1999–2010) NHANES[1]

	Serum folate cutoff, nmol/L (time period)			RBC[2] folate cutoff, nmol/L (time period)		
Population group	<10[3] (1988–1994)	<10[3] (1999–2006)	<14[4] (2007–2010)	<340[3] (1988–1994)	<340[3] (1999–2006)	<624[4] (2007–2010)
Age group, y						
4–11	7.8 (6.3, 9.5)	<1[†]	<1[†]	20 (16, 23)	0.9 (0.7, 1.3)	2.1 (1.5, 3.0)
12–19	38 (35, 42)	1.2 (0.9, 1.7)	2.1 (1.4, 3.1)	49 (45, 54)	6.3 (5.4, 7.3)	11 (8.8, 14)
20–39	47 (44, 50)	2.6 (2.2, 3.1)	6.7 (5.7, 8.0)	46 (43, 48)	7.0 (6.0, 8.0)	12 (11, 14)
40–59	40 (37, 43)	2.4 (2.0, 3.0)	4.9 (4.0, 5.8)	35 (32, 39)	4.5 (3.9, 5.2)	8.7 (7.1, 11)
≥60	23 (21, 26)	1.2 (0.9, 1.5)	2.5 (1.9, 3.3)	24 (22, 26)	2.8 (2.4, 3.4)	4.6 (3.7, 5.5)
Sex						
Male	38 (35, 41)	2.0 (1.7, 2.4)	4.7 (3.9, 5.5)	37 (35, 39)	4.9 (4.2, 5.6)	8.9 (7.8, 10)
Female	33 (31, 36)	1.7 (1.4, 2.0)	3.7 (3.2, 4.3)	37 (34, 40)	4.7 (4.3, 5.2)	8.2 (7.3, 9.3)
Race-ethnicity						
Mexican American	41 (37, 44)	1.6 (1.2, 2.2)	5.4 (4.5, 6.5)	41 (36, 47)	5.0 (4.0, 6.2)	10 (8.2, 13)
Non-Hispanic black	46 (44, 48)	2.9 (2.3, 3.5)	7.7 (6.6, 9.1)	60 (58, 62)	13 (12, 15)	18 (16, 20)
Non-Hispanic white	34 (31, 37)	1.6 (1.4, 1.9)	3.4 (2.8, 4.3)	33 (31, 36)	3.1 (2.6, 3.7)	6.1 (5.1, 7.3)
Supplement use						
Yes	39 (36, 42)	0.8 (0.6, 1.0)	2.0 (1.6, 2.6)	37 (33, 42)	2.2 (1.8, 2.6)	4.0 (3.5, 4.6)
No	34 (31, 37)	2.9 (2.6, 3.3)	6.0 (5.2, 6.9)	37 (34, 40)	7.3 (6.6, 8.0)	12 (11, 14)

[1] All values are percentages (95% CIs). Data were determined on the basis of rising homocysteine as metabolic indicator. Data for 1988–2006 were generated with BioRad radioprotein-binding assay (Bio-Rad Laboratories); data for 2007–2010 were generated with the CDC microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator. [†]Estimate was suppressed; relative SE ≥40%.
[2] RBC, red blood cell.
[3] BioRad radioprotein-binding assay cutoff was used as is.
[4] BioRad radioprotein-binding assay cutoffs of 10 nmol/L (serum folate) and 340 nmol/L (RBC folate) were adjusted to microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator units (7).

Some previous reports used assay-mismatched cutoffs (20–25); sometimes, this practice was intentional because the use of assay-matched cutoffs with postfortification NHANES data produced very-low prevalence estimates for risk of deficiency on the basis of megaloblastic anemia (small cell size) (23). Nonetheless, the use of assay-mismatched cutoffs may have been why prevalence estimates for serum folate were different than those for RBC folate (24). In our report, risks of deficiency were similar for both biomarkers prefortification (serum folate: 5.6%; RBC folate: 7.4%) and postfortification (serum and RBC folate <1%) when we used assay-matched cutoffs. Risks of possible deficiency on the basis of rising Hcy were also similar for both biomarkers prefortification (serum folate: 35%; RBC folate: 37%) and of similar magnitudes postfortification (serum folate: 4.2%; RBC folate: 8.6%). Risk of insufficiency on the basis of elevated NTD risk in women of reproductive age could only be interpreted with the use of RBC folate and it was 23% during the 2007–2010 postfortification period.

TABLE 7
Prevalence for risk of folate insufficiency by population subgroup with the use of assay-matched cutoffs and original data for women aged 12–49 y in the postfortification (2007–2010) NHANES[1]

	RBC[2] folate cutoff (<748 nmol/L[3]) and time period (2007–2010)
Race-ethnicity	
Mexican American	21 (17, 25)
Non-Hispanic black	38 (35, 41)
Non-Hispanic white	18 (16, 21)
Supplement use	
Yes	12 (10, 14)
No	30 (27, 33)

[1] All values are percentages (95% CIs). Data were determined on the basis of elevated risk of neural tube defects. Data for 2007–2010 were generated with the CDC microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator.
[2] RBC, red blood cell.
[3] Microbiologic assay with chloramphenicol-resistant strain and folic acid calibrator cutoff of 906 nmol/L was adjusted to microbiologic assay with chloramphenicol-resistant strain and 5-methyltetrahydrofolate calibrator units (12, 13).

Several expert panels have previously discussed the appropriateness of commonly used cutoffs to estimate the prevalence of low folate status (1, 5, 8, 9, 10, 16). Some of those issues have been summarized (2), and a recent review article discussed general issues related to cutoffs for nutritional biomarkers (26). Because a cutoff of adequacy should discriminate accurately (with few misclassification errors) between healthy and at-risk groups, it is preferred that cutoffs are identified or validated through clinical trials with the use of accurate assays (2, 26). The folate cutoffs for risk of deficiency on the basis of megaloblastic anemia were derived from experimental data (limited sample size) and, therefore, were most closely aligned with this requirement. However, this was not the case for the cutoffs for risk of possible deficiency on the basis of rising Hcy, which were derived from cross-sectional prefortification NHANES data. A validation of these cutoffs through experimental data are lacking and, thus, any resulting prevalence estimates should be interpreted with caution.

Another reason that cutoffs for risk of possible deficiency on the basis of rising Hcy should be used with caution is due to the underlying assay (BioRad RPBA). For whole-blood samples, the

relation between the BioRad RPBA and the MBA-3 depends on the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) *C677T* genotype, whereby RBC folate concentrations in persons with a *T/T* genotype are overestimated and concentrations in persons with a *C/C* genotype are underestimated (27). However, *MTHFR C677T* genotype information is often not available, which necessitates the use of "all-genotype" rather than "genotype-specific" regression equations to adjust the data (7). Issues of inaccurate RBC folate concentrations also appear to be present in other protein binding assays (28). Last, the BioRad RPBA was discontinued ~10 y ago and, to our knowledge, has not been used in other national nutrition surveys. Information on how the BioRad RPBA compares with assays used in other nutrition surveys is missing, thereby making it impossible to suggest appropriate adjustment factors. All of these issues limit the implementation of the WHO cutoffs for possible deficiency on the basis of rising Hcy internationally and raise questions about the US data (1988–2006) specifically for RBC folate. Because of the limitation of the nonexperimental study design and the known biases of the biomarker assay used, it is important to raise these points particularly because some investigators are solely relying on the WHO cutoffs to interpret population folate status.

The idea that adjusting the data compared with adjusting the cutoffs should make no difference in the prevalence was indeed what we observed for risk of possible deficiency on the basis of rising Hcy both prefortification and postfortification when we used the same regression equations to either adjust the data or the cutoffs. However, this was not the case for risk of deficiency on the basis of megaloblastic anemia prefortification (~3-fold overestimation and underestimation for serum and RBC folate, respectively) when we adjusted the data to the MBA-3 but used MBA-1 cutoffs. The regression equations that related the BioRad RPBA to the MBA-3 were generated with the use of serum and whole-blood samples collected in the postfortification period (27, 29) when folate concentrations were much higher than during prefortification. It is unclear whether the regression equations appropriately characterized the relation between these 2 assays at low folate concentrations particularly for serum folate for which we applied a nonlinear (fractional polynomial) equation.

A likely explanation for the discrepancy observed with RBC folate could be that the adjusted RBC folate cutoff for risk of deficiency on the basis of megaloblastic anemia for the BioRad RPBA (<215 nmol/L) may not be perfect because the regression equation was generated with the use of serum samples (10). Thus, our prefortification prevalence with the use of an assay-matched cutoff and original data (7.4%) may not have been accurate, and the comparison to the prevalence with the use of adjusted data (2.0%) may not have been warranted.

A third explanation could be that the use of MBA-1 cutoffs with MBA-3 data is not truly assay matched. It is not certain how the traditional MBA-1, which was used to generate the IOM cutoffs for risk of deficiency on the basis of megaloblastic anemia, is related to the current MBA-3. The downward adjustment of BioRad RPBA Quantaphase II to I was ~35%, and the Quantaphase I assay was supposedly producing comparable results to the MBA-1 (10). Compared with the MBA-3, the Quantaphase II assay produced ~30% (serum folate) and ~45% (RBC folate) lower concentrations (7). In a circular way, these results suggested that the traditional MBA-1 performed similarly to the current MBA-3. Thus, the use of IOM cutoffs for risk of deficiency with MBA-3–measured data should be acceptable.

A minor challenge related to folate cutoffs is the use of different units (i.e., ng/mL compared with nmol/L) (26). The literature has been inconsistent about the use of conversion factors (e.g., 2.266 is typically used on the basis of the molecular weight of folic acid; and 2.178 is sometimes used on the basis of the molecular weight of 5-methyltetrahydrofolate) and whether to round the cutoff (e.g., serum folate concentration of 6.8 compared with 7 nmol/L; RBC folate concentration of 226.5 compared with 227 nmol/L). Cutoffs that fall on the tail of a distribution can produce very different prevalence estimates even with minor changes in a cutoff. To avoid giving inappropriate implied precision, we rounded adjusted cutoffs to integers (Table 1).

In conclusion, our work serves to re-evaluate the folate status of the US population prefortification and postfortification by applying our best understanding about folate cutoffs and describing their limitations. The results show a great potential for misclassification when inappropriate cutoffs are used, particularly at lower folate concentrations that are present during the prefortification time period. Because of the importance of folate status and discussions about folic acid fortification, these findings are relevant nationally and internationally. Although some questions remain, we can provide answers regarding the use of appropriate cutoffs in the NHANES. However, our findings cannot be generalized to data from clinical assays or from laboratory-developed tests because the relation of those assays to the assays underlying the cutoffs is unknown. There is a need for standardization of folate assays (30). In addition, the overreliance on dichotomous cutoffs has limitations (26) beyond the ones discussed here, and exploring alternative approaches to categorize population folate status, such as the use of multiple risk categories (13), would be beneficial.

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