Folate status in women of reproductive age as basis of neural tube defect risk assessment

Lynn B. Bailey and Dorothy B. Hausman
Department of Foods and Nutrition, College of Family and Consumer Sciences, University of Georgia, Athens, Georgia
Address for correspondence: Lynn B. Bailey, Ph.D., Department of Foods and Nutrition, College of Family and Consumer Sciences, University of Georgia, Athens, GA 30602. folate@uga.edu

Reliable folate status data for women of reproductive age (WRA) to assess global risk for neural tube defects (NTDs) are needed. We focus on a recent recommendation by the World Health Organization that a specific “optimal” red blood cell (RBC) folate concentration be used as the sole indicator of NTD risk within a population and discuss how to best apply this guidance to reach the goal of assessing NTD risk globally. We also emphasize the importance of using the microbiologic assay (MBA) as the most reliable assay for obtaining comparable results for RBC folate concentration across time and countries, the need for harmonization of the MBA through use of consistent key reagents and procedures within laboratories, and the requirement to apply assay-matched cutoffs for folate deficiency and insufficiency. To estimate NTD risk globally, the ideal scenario would be to have country-specific population-based surveys of RBC folate in WRA determined utilizing a harmonized MBA, as was done in recent studies in Guatemala and Belize. We conclude with guidance on next steps to best navigate the road map toward the goal of generating reliable folate status data on which to assess NTD risk in WRA in low- and middle-income countries.

Keywords: global; RBC folate; NTD risk; women; reproductive age

Introduction

Here, we focus on red blood cell (RBC) folate concentration and the rationale for the selection of this key biomarker by the World Health Organization (WHO) as a validated indicator of (neural tube defect) NTD risk within a population. The issue of whether serum (or plasma) folate concentration may also be used to assess NTD risk is addressed. We present a synopsis of the evidence base for the selection of these biomarkers as the key indicators of folate status, including their responsiveness to changes in folate intake, the timeline for the response, reflection of body folate stores, and whether they can be used to assess folate status in population groups. The importance of considering how selected biological and environmental determinants of folate status may affect biomarker response is also highlighted. Folate status is considered in relation to “optimal” blood folate concentration in women of reproductive age (WRA) associated with lowest NTD risk. Evidence is presented that cutoffs for folate deficiency that are based on the prevention of megaloblastic anemia cannot be used to define the optimal blood folate for NTD risk reduction, as considerably higher concentrations of folate are required to support rapidly dividing cells during early embryonic development. Hence, the term folate insufficiency has been adopted to describe RBC folate concentrations within a population group that are below the cutoff for optimal RBC folate concentration in WRA (Table 1).

[Correction added on March 6, 2018, after first online publication: Reference 67, Rogers, L.M., A.M. Cordero, C.M. Pfeiffer, et al. 2018, was removed from the reference list and the in-text citations were changed from “67” to “(unpublished data)”. Reference 68 was renumbered as Reference 67 and the in-text citations were renumbered accordingly.]
Table 1. Folate status cutoffs and basis for definition

| Definition      | Basis for definition                     | Serum folate (nmol/L) | RBC folate (nmol/L) |
|-----------------|------------------------------------------|-----------------------|---------------------|
| Deficiency      | Hematologic indicator: megaloblastic anemia | $<7^a$ | $<227^a$ |
|                 | Hematologic indicator: risk of megaloblastic anemia | $<7^b$ | $<305^b$ |
| Possible deficiency | Metabolic indicator: rising homocysteine | $<10^c$ | $<340^c$ |
| Insufficiency   | Elevated neural tube defect risk          | NA                    | $<906^c$ |
|                 |                                          | NA                    | $<748^d$ |

$^a$Presented in the 2012 WHO report$^1$ as “values indicative of folate deficiency, on the basis of the concentrations at which megaloblastic anemia is more likely to appear”; folate analysis by MBA with wild-type strain and folic acid calibrator. No adjustment necessary when cutoff is applied to data generated with the CDC MBA (chloramphenicol-resistant strain and 5-methyl-THF calibrator).$^{19}$

$^b$Presented in the IOM report$^{67}$ as “negative folate balance” for serum folate and “appearance of hypersegmented neutrophils” for RBC folate; folate analysis by MBA with wild-type strain and folic acid calibrator.

$^c$Presented in the 2012 WHO report$^1$ as “measured by the radioimmunoassay” and needing “adjustment to make [cutoffs] comparable with the microbiologic assay.”

$^d$Adjusted cutoff to be used with data generated with the CDC MBA (chloramphenicol-resistant strain and 5-methyl-THF calibrator).$^{19}$

$^e$Presented in the 2015 WHO report$^1$ as “folate concentrations in red blood cells preventing neural tube defect–affected pregnancies in women of reproductive age at the population level”; folate analysis by MBA with chloramphenicol-resistant strain and folic acid calibrator.

concentration is summarized, including the fact that the microbiologic assay (MBA) is considered the most reliable assay for obtaining comparable results for RBC folate concentration across time and countries.$^1$ Although it is the recommended procedure for RBC folate assessment, the use of alternate conditions in the conduct of the MBA in different laboratories can lead to different results;$^2$ thus, the cutoff used to define insufficiency needs to be adjusted to account for assay differences. Furthermore, for the global assessment of NTD risk, there is a need to harmonize the MBA through the use of consistent key reagents and procedures, standardized training protocols, and appropriate quality control measures in selected laboratories conducting the RBC folate analysis. Of critical importance is the establishment of a framework for laboratory harmonization of folate analytical measurements, a topic addressed elsewhere.$^3$ Recent studies conducted in Guatemala and Belize are highlighted in this paper to illustrate how the WHO guideline can be appropriately applied to assess NTD risk on the basis of RBC folate concentration in the most vulnerable population groups of WRA.

Background: folate status biomarkers

Evidence related to the selection, use, measurement, and interpretation of key folate status biomarkers, including the genetic, biological, and sociodemographic determinants, has been extensively reviewed.$^{1,4,5}$ Serum/plasma and RBC folate concentrations have been identified as valid biomarkers of folate status of individuals and populations and of folate status in relation to NTD risk in WRA.$^{1,4,5}$ In addition, estimating dietary and supplemental folic acid intake is an informative component of comprehensive folate status assessment. Methodological issues pertaining to the estimation of dietary folate and supplemental folic acid intake and folate biomarkers were reviewed in 2015.$^5$

Serum folate concentration reflects recent dietary intake, is the earliest indicator of modified folate exposure, and is an informative biomarker of short-term folate status.$^{5,6}$ Although a single measurement of serum folate cannot distinguish between a transient change in dietary folate intake and chronically decreased status, repeated measures over time in the same individual can reflect sustained folate status. Serum folate may reflect seasonal availability of folate-rich foods.$^{5,7}$ Population data indicate that serum folate concentrations are highly reflective of the degree of exposure to folic acid, with the highest levels associated with intake of both fortified foods and supplements.$^{5–13}$ Results of numerous studies, including controlled intake trials in WRA,$^{14}$ large-scale intervention trials,$^{15,16}$ and a recent meta-analysis of randomized controlled trials,$^{17}$ indicate a linear, dose-dependent increase in serum folate in...
response to intervention with folic acid. Thus, serum folate concentration is very responsive to intervention, with folic acid inducing a better response compared with natural food folates at similar intervention levels.\textsuperscript{5,18} In nationally representative surveys, such as the National Health and Nutrition Examination Survey (NHANES), serum folate can be used to define a percentage of the total population or specific population subgroups at risk of folate deficiency.\textsuperscript{19}

RBC folate concentration is a sensitive biomarker of longer term (months) folate status and is the only biomarker that has been linked directly to NTD risk.\textsuperscript{3,20,21} It reflects the amount of folate taken up by developing reticulocytes during erythropoiesis in the bone marrow\textsuperscript{22} and released into the circulation in mature RBCs with impermeable cell membranes. As the concentration within each mature RBC remains the same for the duration of its 120-day life span, RBC folate concentration is generally considered to reflect folate status during the preceding 3–4 months.\textsuperscript{23,24} Consequently, although RBC folate concentration is responsive to intervention with folic acid,\textsuperscript{9,13,17} trial periods of 6–9 months or more may be required to achieve a steady-state RBC folate response to folic acid intervention.\textsuperscript{15,25} As for serum folate, population data indicate that RBC folate concentrations are reflective of the degree of exposure to folic acid, with the highest levels associated with intake of both fortified foods and supplements.\textsuperscript{8,9,13} In addition, as for serum folate, folic acid typically induces a greater RBC folate response compared with natural food folates at similar intervention levels.\textsuperscript{5,18}

The primary storage organ for folate is the liver, accounting for about 50% of total body folate.\textsuperscript{26} On the basis of folate analyses of liver biopsies, RBC folate concentration parallels liver folate content, with decreases in liver folate content reflected in decreases in RBC folate concentration.\textsuperscript{26} As required for DNA synthesis and methylation for regulation of cell synthesis and growth, folate is released from the liver into the serum and transported throughout the body. When liver stores become depleted, hematological changes indicative of the early stages of clinical deficiency occur, which if sustained will result in megaloblastic anemia. Depletion of liver stores, as reflected in the biomarker for liver stores (RBC folate concentration), will also have a major impact on embryonic development. Since the rate of cell division is markedly accelerated during embryogenesis when the neural tube is forming, adequate folate must be available to meet the increased demands for DNA synthesis and subsequent cell growth and development. When considering which biomarker to use to assess NTD risk for population groups of WRA, what is needed is an indicator of body stores of folate that supply the developing embryo when the neural tube is forming. The fact that RBC folate concentration reflects liver folate content and body folate stores\textsuperscript{26} provides support for its selection as the best indicator of sufficient folate to reduce the risk of folate-sensitive NTDs.

Numerous biological and contextual factors affecting serum and RBC folate concentrations were discussed in a 2015 review by Bailey et al.\textsuperscript{5} Of these, we highlight nutrient interactions, genetics, endemic infectious disease, environmental toxins, and socioeconomic status/ethnic/cultural differences as key factors to consider when assessing folate status in low- and middle-income countries (LMICs).

Nutrients, including vitamin B\textsubscript{12}, vitamin B\textsubscript{6}, iron, and riboflavin, have been shown to influence folate metabolism and status.\textsuperscript{5} Of these, vitamin B\textsubscript{12} is most intimately associated with folate function, as it serves as a coenzyme in the methionine synthase reaction required for the conversion of 5-methyltetrahydrofolate (5-methyl-THF) to THF. Accordingly, a vitamin B\textsubscript{12} deficiency traps 5-methyl-THF, thus reducing the regeneration of THF required for DNA synthesis, which may result in a secondary folate deficiency and impair cell division. Low RBC folate concentrations and increased risk for NTD have been reported for WRA characterized as vitamin B\textsubscript{12} deficient or marginally deficient in several studies.\textsuperscript{27,28} This association is particularly important in LMICs, where vitamin B\textsubscript{12} may be limited in the diets of WRA. The impact of folate–vitamin B\textsubscript{12} interaction on risk of NTDs is discussed in greater detail elsewhere.\textsuperscript{29}

Several single-nucleotide polymorphisms have been investigated in regard to folate status,\textsuperscript{5} with the greatest effects observed for MTHFR 677C→T, which reduces the activity of methylenetetrahydrofolate reductase (MTHFR), a rate-limiting enzyme in folate-mediated one-carbon metabolism.\textsuperscript{5} Distribution of the MTHFR 677C→T polymorphism varies by geographic region and race/ethnicity.\textsuperscript{30,31} MTHFR genotype is associated with NTD risk in a
dose-related manner, with the relative risk greatest for those with the TT as compared with the CT and CC genotypes.\textsuperscript{32–34} Although a serum and RBC response to folic acid supplementation has been demonstrated for all \textit{MTHFR} genotypes,\textsuperscript{31,32} higher levels of folic acid supplementation may be required to increase RBC folate to specific target concentrations in those with the TT genotype owing to lower baseline folate concentrations.\textsuperscript{32}

In many developing countries, it is important to consider the potential impact of disease, particularly malaria, on folate status when utilizing these biomarkers for NTD risk assessment. Some reports indicate that malarial infection may induce folate deficiency owing to inadequate intake, malabsorption, increased folate use, and antimalarial drugs.\textsuperscript{35–37} In contrast, other evidence indicates that RBC and serum folate concentrations are elevated in association with malaria,\textsuperscript{36,38,39} with such increases attributed to a potential predisposition to malaria in subjects with high RBC folate, an artificial elevation of RBC folate due to \textit{de novo} folate synthesis by malarial parasites, and increased hemolysis of folate-rich RBCs with resultant increase in serum folate. Some antimalarial drugs may also influence folate status, as they are among the wide range of drugs classified as antifolates. Although a deterioration of folate status has been reported with high doses of antifolate antimalarial drugs,\textsuperscript{36,37} the impact of these drugs at currently recommended lower doses remains to be determined.\textsuperscript{1} Thus, folate biomarker concentrations in association with malaria may be influenced by both prophylactic/therapeutic treatment and the infection itself.\textsuperscript{1,33–39} To minimize these influences, it has been suggested that assessments of folate status not be conducted during periods of peak parasitic load immediately after febrile episodes.\textsuperscript{1}

Contamination of ground water and food products by environmental toxins is highly prevalent in LMICs. Some of these toxins are reported to be teratogenic and may negatively interact with folate metabolism. For example, exposure to low doses of arsenic from contaminated drinking water and food can decrease the efficacy of folic acid in NTD prevention.\textsuperscript{40} Nuclear folate–dependent \textit{de novo} thymidylate biosynthesis has been demonstrated to be a sensitive target of arsenic, providing a mechanism for arsenic in the etiology of developmental anomalies.\textsuperscript{41} As nutrition status may explain individual differences in susceptibility to arsenic toxicity, there is a particular need for public health interventions targeting folate and other micronutrient deficiencies in arsenic-exposed regions.\textsuperscript{12,43}

Socioeconomic variables associated with poverty are recognized to influence folate status,\textsuperscript{44} such as in Guatemala, where the national prevalence of folate insufficiency among WRA is approximately 50\%, but varies considerably by region and socioeconomic characteristics,\textsuperscript{45} as discussed below. In Belize, ethnicity rather than socioeconomic status was the primary predictor of folate status, with the highest prevalence of folate insufficiency observed among women of Mayan origin,\textsuperscript{27} who were less likely to consume fortified wheat products owing to cultural preferences.

There are many biological and contextual factors, including those described above, that may influence folate status.\textsuperscript{3} Nonetheless, the largest contributing factor to folate status and body stores is likely folic acid intake.

**RBC folate as a basis for NTD risk assessment**

In 2015, the WHO established a guideline for optimal blood folate concentration in WRA for optimum prevention of folate-sensitive NTDs.\textsuperscript{1} This guideline establishes a threshold for RBC folate concentration at the population level that can be used to determine the need for and guide monitoring and evaluation of the impact of nutrition interventions designed to enhance folate status and reduce NTD risk.

The WHO guideline and key conclusions are as follows:

1. An “optimal” RBC folate concentration should be exceeded to achieve the greatest reduction in folate sensitive NTDs. An “optimal” RBC folate concentration, defined as 906 nmol/L, should be used to assess folate insufficiency in population groups of WRA but not to assess NTD risk of individuals. This threshold value is not a mean or median value but is the lowest end of the RBC folate distribution above which all women in the population should exceed to achieve optimal NTD risk reduction.
2. The assay of choice to measure RBC folate is the MBA.
3. No serum folate threshold is recommended for the prevention of folate sensitive NTDs.

The primary basis of the WHO guideline for optimal blood folate for NTD risk reduction was an Irish study for which blood samples were collected for case–control analysis of NTDs. Of the >56,000 first antenatal visit samples collected at three major hospitals in Dublin, 84 were from women who subsequently gave birth to an NTD-affected child (cases). A systematic sample of women with normal pregnancy outcome \( n = 242 \); three per case served as controls to determine the relationship between prenatal RBC folate concentration and NTD-affected birth outcomes. Since RBC folate concentration reflects folate status \( \sim 120 \) days earlier, measurements made in this study at a median of 15 weeks gestation were considered indicative of early gestational folate status when neural tube closure occurred. An inverse relationship between RBC folate and NTD risk was documented throughout the distribution of values with very high NTD risk (6.6 per 1000 births) associated with RBC folate concentrations \(<340 \) nmol/L. NTD risk decreased throughout the continuum of RBC folate concentrations until the threshold value (906 nmol/L) was attained (Fig. 1). This concentration, associated with the lowest NTD risk, was much higher than concentrations that previously had been considered as normal (on the basis of the risk of megaloblastic anemia). These findings provide evidence that the earlier cutoffs for folate deficiency cannot be used to define the optimal blood folate for NTD risk reduction. For this reason, the term folate insufficiency has been adopted to describe an RBC folate concentration within a population group that is below the WHO cutoff for optimal RBC folate concentration (i.e., 906 nmol/L).

A Bayesian modeling approach using data from two folic acid intervention studies in China was used by Centers for Disease Control (CDC) investigators to estimate the association between maternal RBC folate concentration at the time of neural tube closure and NTD risk. The predicted optimal RBC folate concentration for NTD risk reduction (Fig. 1; \(~1000 \) nmol/L) was remarkably consistent with the threshold observed in the Irish study, which was the basis of the WHO guideline.

This modeled association was then used to predict NTD risk in the United States before and after fortification on the basis of population-based RBC folate. Because of methodological differences in the MBAs used in the two studies, before modeling it was necessary to normalize the NHANES data to the RBC folate concentration distributions of Daly et al. using the equation published by Pfeiffer et al. Using this modeling approach, the prevalence of NTD was estimated to be in the range of 1.01–1.6 per 1000 births during the prefortification period and in the range of 0.4–0.8 per 1000 births during the postfortification period, consistent with published prevalence estimates. The fact that these estimates of NTD risk based on RBC folate concentrations are comparable between very different populations substantiates the potential importance of RBC folate concentration as a biomarker for NTD risk. This consistency in the estimated optimal RBC folate concentration predictive of NTD risk also supports the generalizability of this biomarker across different populations and exposures to folic acid. Nonetheless, there are limitations of the Crider et al. model when applied to U.S. populations of non-Hispanic blacks (NHBs), who have lower folate concentrations and lower NTD rates relative to other racial/ethnic groups, which has been proposed to reflect the fact that a genetic mutation negatively affecting folate metabolism is less prevalent in NHBs relative to other racial/ethnic groups in the United States.

A key question is whether the maximum reduction in NTDs can be attained by optimizing RBC folate concentration. In this regard, it is important to note that not all NTDs are folic acid sensitive, as evidenced by multiple studies that report the lowest observed NTD prevalence in response to periconceptional folic acid supplementation and food fortification programs to be 0.5–0.6 per 1000 births. According to both Daly et al. and the Crider modeling study, the risk of NTDs decreased as RBC folate concentration increased at the same dose–response relationship in both studies. The results from the Crider et al. investigation indicate that an RBC folate concentration of \(~1000 \) nmol/L (assay adjusted) is associated with the maximum prevention of folate-sensitive NTDs, with a resulting overall NTD risk of \(~0.6 \) per 1000. On the basis of this body of evidence, it is reasonable to conclude that the lowest prevalence of NTDs...
Obtainable through folic acid–based interventions is ~0.5–0.6 per 1000 births.

Evidence from the Daly et al. study and the NTD prediction modeling approach support the conclusion that relatively modest increases in RBC folate concentrations among women with the lowest concentrations result in the greatest NTD risk reduction. For example, in the United States, the estimated NTD risk for women in the fifth percentile of RBC folate concentration decreased from 3.6 (prefortification) to 1.5 (postfortification) per 1000 births with a relatively moderate increase in RBC folate concentration. The estimated daily intake of folic acid from enriched cereal grain products associated with this > twofold reduction in NTD prevalence was only 138 μg/day, much lower than the recommended 400 μg/day to reduce NTD risk. These findings provide evidence that WRA with the lowest RBC folate concentrations are likely to be at the highest risk for NTD-affected pregnancies and are most likely to be positively affected by modest and sustained intakes of folic acid, such as that provided in a fortified staple food. Accordingly, folic acid food fortification programs are predicted to be most effective in affecting NTD risk in the most vulnerable WRA who have the lowest RBC folate concentrations. From a public health perspective, all WRA are at risk of an NTD-affected pregnancy if they are folate insufficient, and the risk is driven mostly by low folate status at the tail of the distribution.

In summary, at the population level, the WHO recommends that RBC folate concentrations should be above 906 nmol/L in WRA to achieve the greatest reduction of NTDs. This recommendation is based on data from an Irish NTD case-controlled study in which NTD risk rapidly decreased with increasing RBC folate with a threshold concentration (906 nmol/L) above which NTD risk was lowest and confirmed by a data modeling approach that used data from different populations. When assessing folate insufficiency within a population group, it is important to note that the threshold value from the WHO guideline is not a mean or median value but is the lowest end of the RBC distribution for the population above which folate-sensitive NTD risk reduction is optimal (i.e., in the United States, 25% of WRA have RBC folate falling below the threshold; in Guatemala and Belize (see below), ~50% fall below the threshold). Furthermore, all individuals falling below this cutoff are not at equal NTD risk. Thus, data must be examined across distribution ranges or percentiles of RBC folate concentration as discussed later in this paper.

Serum/plasma folate concentration: rationale for exclusion as a basis for NTD risk assessment

Serum or plasma folate concentration is routinely measured in nutritional assessment of populations and is the sole biomarker for folate deficiency in many surveys to date. This fact raises the important
question of whether serum/plasma folate could be used to assess risk for NTDs. In the Daly et al. study, serum folate was measured and included in the report but was not used to determine an optimal threshold concentration for NTD risk reduction. There were three reasons given as rationale for only considering RBC folate to predict NTD risk in this case–control trial: (1) the samples were drawn from pregnant women whose serum/plasma folate may have been significantly affected by increases in plasma volume resulting in hemodilution of the reported values obtained at ~15 weeks gestation; (2) serum/plasma folate (unlike RBC folate) is sensitive to changes in recent dietary intake of folate and may not reflect body stores and thus the amount available to the developing embryo; and (3) serum/plasma folate concentrations would more likely reflect status at the time the samples were drawn (~15th week of gestation) in contrast to RBC folate, which is known to reflect folate status during the past 120 days, encompassing the time of embryonic neural tube closure. The WHO committee did not recommend an optimal serum folate concentration to assess NTD risk. Countries interested in using serum folate concentration to estimate NTD risk were advised to first establish the relationship between serum and RBC folate and then use the optimal value for RBC folate for NTD risk reduction to determine the corresponding threshold for serum. Optimal threshold concentrations of serum/plasma folate were likewise not determined in the modeling study of Crider et al., which concluded that “additional research is needed to explore the utility of using serum/plasma folate concentrations to predict the risk of NTDs and to monitor prevention programs.”

Since many countries have reported serum/plasma folate concentrations as the sole folate status biomarker or have stored serum/plasma samples that could be analyzed, it is important to consider whether these data could be useful in assessing NTD risk. It may be reasonable to advise countries to compare serum/plasma folate concentrations already available with that reported by other countries that have also measured RBC folate (if the same analytical method was used). Such comparisons are most appropriate for folate data obtained using the MBA, which, unlike protein-binding and chromatographic assays, can be adjusted using assay-specific cutoffs (see below). When comparisons are appropriate on the basis of methodological considerations, it may be possible to draw conclusions as to next steps based solely on serum/plasma folate concentrations. For example, if serum/plasma folate concentrations in an LMIC are comparable to those in the United States postfortification (i.e., mean values ~40 nmol/L), it might be reasonable not to pursue a folic acid intervention program but to incorporate RBC folate measurements in future population surveys. In contrast, as the cutoff for deficiency is approximately one-third of the cutoff for insufficiency associated with NTD risk, the presence of serum/plasma folate concentrations indicative of folate deficiency (megaloblastic anemia risk indicator, serum/plasma folate ≤7 nmol/L) in a country would suggest a much greater prevalence of RBC concentrations below the insufficiency threshold. In such cases, an immediate folic acid intervention should be initiated on the basis of serum folate status indicators, with RBC folate measurements planned at baseline and postintervention.

**Microbiologic assay: most reliable method to quantify RBC folate and the need to harmonize assays and use assay-matched cutoffs of folate insufficiency**

Analytical methods and technical issues related to the measurement of blood folate concentration were extensively reviewed by Bailey et al. “Folate” comprises a group of folate forms or derivatives with vitamin activity, which results in more analytical challenges than for many other vitamins. Preanalytical, analytical, and postanalytical challenges of folate measurement are detailed elsewhere. The three analytical techniques primarily used for the measurement of folate are based on the MBA, protein-binding assays, and chromatographic assays. Each technique has advantages and disadvantages, but only the MBA combines the features that make it appealing for use for surveys in LMICs: it requires only a small specimen volume, measures all active forms of folate in the sample, requires rather simple instrumentation, and is fairly inexpensive. Considering the advantages and disadvantages of the various analytical techniques, the WHO recommends the MBA as the “most reliable choice to obtain comparable results for RBC folate across countries” for the assessment of NTD risk within a population.

Although the MBA is the recommended procedure for RBC folate assessment, the use of
different microorganisms or folate calibrators in the conduct of this assay can lead to different results and cutoffs for risk of insufficiency.\textsuperscript{2,19–21,58} The WHO recommendation of RBC folate concentration \textgreater{} 906 nmol/L as a cutoff for optimal folate status for NTD risk reduction in WRA is based on data obtained using the contemporary MBA (chloramphenicol-resistant strain of \textit{Lactobacillus rhamnosus}) calibrated with folic acid.\textsuperscript{20,59} This assay generates higher RBC folate concentration data than the contemporary MBA calibrated with 5-methyl-THF as used in NHANES. Thus, for data obtained with the latter assay, a cutoff of \textgreater{} 748 nmol/L is used to define optimal RBC folate concentration for NTD risk reduction.\textsuperscript{58} To allow the comparison of MBA data obtained using different calibrators, a conversion formula can be applied.\textsuperscript{2} Alternatively, an assay-matched cutoff\textsuperscript{19} can simply be used to define folate insufficiency in a population as \textless{} 906 nmol/L for MBA with folic acid calibrator or \textless{} 748 with conventional MBA with a 5-methyl-THF calibrator (Table 1). Furthermore, for the global assessment of NTD risk, there is a need to harmonize the MBA through the use of consistent key reagents and procedures (i.e., the same microorganism (chloramphenicol-resistant \textit{L. rhamnosus}), folate calibrator (5-methyl-THF), standardized training procedures, and appropriate quality control measures (proficiency certifications)) in selected laboratories throughout the world conducting the RBC folate analysis.

In addition to harmonizing MBA assay conditions and using assay-matched cutoffs, the global assessment of folate status in WRA requires simplified data analysis and modeling approaches and greater consistency in the presentation of data to allow comparisons across countries. Modeling approaches used in some of the key studies detailed in this report\textsuperscript{21,45} are complex and may be challenging to utilize extensively. Further, RBC folate concentrations are variably presented as mean, geometric mean, or median values along with or in lieu of the prevalence of deficiency/insufficiency in published survey reports.\textsuperscript{60–62} This is inconsistent and may be misleading. For example, comparison of median values with the 906 or 748 nmol/L cutoffs in populations with a high degree of variation in RBC folate concentrations could lead to a failure to detect a large proportion of WRA in the lower end of the distribution who are at risk of NTD-affected pregnancies. Furthermore, although \textless{} 906 and \textless{} 748 nmol/L have been designated as the assay appropriate cutoffs for greatest NTD risk reduction, not all individuals falling below this cutoff are at equal risk of having an NTD-affected pregnancy. Accordingly, it may be more informative to present the data across distribution ranges for RBC folate concentration, as was done by Daly \textit{et al}.,\textsuperscript{20} who reported the risk of NTD per 1000 births as 6.6 for an RBC folate concentration of 0–339 nmol/L and 1.6 for an RBC folate concentration of 680–906 nmol/L. Presentation of aggregate and/or categorical RBC concentrations by percentile distribution, as was done by Rosenthal \textit{et al}.,\textsuperscript{45} may also be useful for identifying subpopulations most at risk of folate insufficiency.

**Assessing NTD risk with population-based RBC folate concentration measured by harmonized microbiologic assay: key studies**

The WHO guideline can be used to estimate the percentage of WRA in a population who are folate insufficient and thus at increased risk for NTD-affected pregnancies, when the surveys are population based and the MBA was used to measure RBC concentration. To use the WHO guideline to estimate NTD risk in LMICs, the ideal scenario would be to have country-specific, population-based surveys of RBC folate concentrations in WRA determined with a harmonized MBA. Two studies conducted in developing countries (Guatemala and Belize) are highlighted as key examples of population-based surveys that generated data that have been appropriately used to estimate the percentage of WRA who have insufficient RBC folate concentrations for optimal folate-sensitive NTD prevention.

Rosenthal \textit{et al}.,\textsuperscript{45} were the first investigators to apply the WHO guideline for folate insufficiency in an LMIC. Guatemala has a mandatory fortification program for wheat flour, with the amount increased in 2003 from 1.08 to 1.80 mg/kg. The survey (Guatemala Encuesta Nacional de Micronutrientes (ENMICRON)), which can serve as a model for assessing folate insufficiency and NTD risk in other vulnerable populations, was a national and regional multistage cluster probability survey among WRA.\textsuperscript{63} The design of this survey ensured sufficient sample size and population coverage to provide representative estimates of RBC folate.
concentrations for WRA at the national and regional levels. The blood samples were collected, processed, stored, and shipped appropriately to protect blood folate before MBA analysis at the CDC. The assay-matched cutoff <748 nmol/L was used to account for MBA methodological differences. Prevalence estimates of RBC folate insufficiency were determined using prevalence risk ratios for area type (urban or rural) and other biological (i.e., age and ethnicity) and socioeconomic status (SES) (i.e., level of education and wealth index) factors and region. The complex statistical approaches used to estimate NTD risk based on RBC folate concentrations were described previously. 

Results indicated that folate insufficiency among Guatemalan WRA was 47% nationally, showed wide variation (18–81%) by region, and was most prevalent in those with the lowest SES living in rural areas. In all regions, folate insufficiency was higher among indigenous than nonindigenous populations. Using a Bayesian modeling approach, national NTD risk based on RBC folate concentrations was estimated to be 1.4 per 1000 live births and showed wide regional variation (from 1.1 to 2.6 NTDs per 1000 in the Metropolitana and Norte regions, respectively). These findings were consistent with a previous investigation in Guatemala, which determined that folic acid fortification contributed to the estimated average requirement for folate intake in 78% of urban WRA versus only 15% in rural areas and that WRA who lacked the means to purchase fortified staples (e.g., those living in poverty or extreme poverty) were found to be the most vulnerable to inadequate folate intake. Although folic acid fortification has been mandatory for many years, ~50% of WRA in Guatemala are folate insufficient, and it was concluded that folate insufficiency is a common problem in populations with limited access to fortified foods, such as rural, low-income, and indigenous populations. In contrast, in subpopulations of WRA who were most likely to have access to and consume folic acid–fortified wheat flour, the prevalence of folate insufficiency is similar to that of countries with well-established fortification programs.

The significance of this population-based study is that it is the first investigation that assessed folate insufficiency and predicted NTD risk on the basis of the WHO guideline in an LMIC. This study provides an excellent example of the importance of considering factors such as area (rural versus urban) and SES when assessing the efficacy of an existing intervention. This study is also informative with reference to the selection of the staple food product to fortify. In Guatemala, ethnic groups defined as indigenous are most likely to have the lowest wealth level and retain dietary practices that can influence whether fortified staples will be sought out and accepted. Since indigenous populations in Guatemala have a corn-based diet (tortillas), and because they grow their own corn or buy corn to process into corn masa flour locally, it is possible that they are less likely to purchase and consume folic acid–fortified wheat flour.

In the population-based Belize National Micronutrient Survey in WRA, the WHO guideline for optimal RBC folate concentration was appropriately applied (RBC folate concentration analyzed with harmonized MBA and assay-matched cutoffs used) to assess NTD risk on the basis of the prevalence of folate insufficiency. 

Fortification is currently voluntary in Belize, and this study was designed to provide a baseline assessment of folate status before establishing a mandatory fortification program. National prevalence of folate insufficiency was estimated to be 49% on the basis of the assay-matched optimal blood folate concentration (>748 nmol/L). This finding is comparable to that observed in Guatemala and provides evidence that ~50% of the population of WRA in these two countries are at risk of having a folate-sensitive NTD-affected pregnancy. As in Guatemala, there were significant differences between the prevalence of folate insufficiency among different subgroups of WRA in Belize (ranging from 33.5% to 68.7%). The high prevalence of insufficiency in some subgroups is likely because the indigenous poor have restricted access to fortified food, limited purchasing power, and/or do not consume wheat flour as their main food staple. This is an important consideration for countries, such as Belize, which are planning to implement mandatory fortification. Of key importance is the necessity for the folic acid–fortified food products to be an accessible, affordable, and acceptable dietary staple in the at-risk population groups. Expanded fortification programs that include additional staples, such as corn flour and/or rice, may be needed to reach at-risk population groups of WRA to reduce folate insufficiency and prevent folate-sensitive NTDs. New targeted intervention
efforts are needed to reach the high-risk regions and
the most vulnerable populations to increase folic
c acid intake and RBC folate concentrations. Mandatory
fortification programs should be considered,
as they have the potential to be more effective than voluntary programs, which may miss portions of
the population most in need of improvement in folate status, as detailed in a separate paper.65

The findings from these studies are of special
interest in relation to establishing a road map for
NTD risk reduction on the basis of RBC folate con-
centrations. In addition to defining folate insuffi-
ciency and estimating NTD risk, information about
current folic acid fortification programs, including
whether existing programs are mandatory, the spe-
cific staple(s) fortified, the amount of folic acid in
the staple, and the contribution to the daily folate
intake of WRA where possible, would best inform
appropriate next steps.

Global folate status in women
of reproductive age

An important step in evaluating current global folate
sufficiency as a basis of NTD risk assessment is
to review available data regarding folate status in
LMICs. A review published in 200866 provided an
indication of the prevalence of folate deficiency in
various regions or countries, but, owing to the lack
of nationally representative, population-based data,
did not allow conclusions to be drawn regarding the
extent of folate deficiency worldwide. To provide
an updated (2000–2016) and more focused account
of global folate status of WRA, a systematic review
was recently undertaken by the CDC and the WHO
to select folate status surveys that meet inclusion
criteria (e.g., population-based, WRA, survey year ≥ 2000). The results of this review, which include
information regarding folate analytical methodol-
gy along with other survey characteristics, are the
focus of a separate paper (unpublished data).

Summary and recommendations

Much work has been accomplished toward the goal
of generating reliable folate status data with which
to assess NTD risk. A framework for global assess-
ment of folate status in WRA was provided by the
WHO report of 2015,1 which recommended
RBC folate concentrations and the MBA assay as
the preferred folate biomarker and analytical assay,
respectively, for assessing folate status. The report
also established a threshold for optimal RBC folate
(>906 nmol/L) that can be used at the population
level to determine the need for and guide moni-
toring and evaluation of the impact of nutrition
interventions designed to enhance folate status and
reduce folate-sensitive NTD risk. Consistency in the
estimated optimal RBC folate concentration pre-
dictive of NTD risk in the original study on which
the WHO recommendation was based and in sub-
sequent modeling studies in other populations sup-
ports the generalizability of this biomarker across
populations with different racial groups and expo-
sure to folic acid. Furthermore, it was noted that for
data obtained with the contemporary MBA using
5-methyl-THF rather than folic acid as the assay
calibrator, an optimal RBC folate concentration
for folate-sensitive NTD risk reduction was deter-
mined to be >748 nmol/L. Therefore, in using the
MBA for determination of RBC folate concentra-
tion as the biomarker for NTD risk assessment, it is
important to apply appropriate assay-matched cut-
offs for determining folate insufficiency. This was
done for recent population-based surveys in two
LMICs, Guatemala and Belize, which indicated that
despite fortification efforts there is an almost 50%
prevalence and high demographic and regional vari-
ation in folate insufficiency in these countries, with
the highest prevalence in poor, rural, and indige-
nous subpopulations.

Gaps in knowledge and needs

Despite the progress to date toward reliable assess-
ment of NTD risk at the population level based
on population distributions of RBC folate concen-
tration, several questions and gaps in knowledge
remain, including the following.

Global harmonization of folate analyses

WHO guidelines recommend the MBA as the most
reliable choice to measure RBC folate concentra-
tion as a basis of assessing NTD risk in a population.
Many studies assessing folate status have either used
an alternative biomarker (serum or plasma folate) or
had differences in the analytical methods. Because
of these methodological issues, there is a critical
need to develop a framework for laboratory harmo-
nization of folate analytical measurements, includ-
ing the use of common critical reagents, trained
laboratory personnel, development of MBA assay
kits, and folate certification programs for MBA assessment.

**Global assessment of folate status**
Efforts are needed to fill gaps in knowledge related to folate status in WRA globally, as detailed in a paper based on a recent systematic review (unpublished data). Folate status assessment pre- and postfortification is important to estimate the efficacy of folic acid fortification programs.

**RBC folate concentration distribution as a basis of NTD risk**
Although assay-matched cutoffs for optimal RBC folate concentration may be useful for determining the overall prevalence of insufficiency, presentation of RBC folate concentration distributions (overall and possibly by subgroups) may also be important to identify subpopulations most at risk. Such data have not been included for most published studies in this area.

**Potential use of serum or plasma folate concentration as biomarker for risk reduction**
Although generally considered a biomarker of short-term folate status at the individual level, studies at the population level indicate that serum folate concentrations are reflective of both the degree of exposure to folic acid and the response to folic acid intervention. Serum folate has been used as the sole biomarker in many published studies from around the world assessing folate status in WRA. However, a threshold for the optimal folate status of populations for NTD risk reduction based on serum concentration has not been established. To use serum folate concentration in the assessment of NTD risk, it is necessary to first establish the relationship between serum folate and RBC folate concentrations and then use the optimal value for RBC folate concentration for NTD risk reduction to determine the corresponding threshold for serum folate concentration.

**Inclusion of dietary information in folate status assessment**
Although not essential for the implementation of a folic acid fortification program, when conducting population-based surveys to determine NTD risk based on RBC folate concentrations, it would be ideal to also include an appropriately performed dietary assessment. This would provide useful information regarding current sources of folate in the diet and food consumption patterns to identify the best foods for fortification. Such an assessment should also provide information about current folic acid fortification programs, including whether existing programs are mandatory, the specific staple(s) fortified, the amount of folic acid in the staple, contribution of fortified foods to the daily folate intake of WRA, and time of implementation of fortification in relation to conduct of folate status assessment, where possible, as to best inform appropriate next steps.

**Appropriate selection of vehicle for fortification**
High prevalence of folate insufficiency and wide variation by regional, socioeconomic, and ethnic subpopulations have been reported in some countries with ongoing folic acid fortification programs. Because of issues related to availability, affordability, and acceptability, wheat and other fortified flours may not be reaching those most vulnerable to low folate status. Furthermore, as cereal staple preferences vary by cultural influences and dietary customs, a single fortified flour product may not be appropriate for an entire country or region. In addition to addressing immediate needs to improve folate status in WRA with established folic acid fortified foodstuffs, fortification of a more universally acceptable food staple, such as condiments, warrants investigation.

**Updated systematic review of vitamin B\(_{12}\) status in LMICs**
The 2008 review by McLean et al.\(^6\) included available data on the global prevalence of both folate and vitamin B\(_{12}\) deficiencies. Data from the recent study in Belize indicate that deficiencies in folate and vitamin B\(_{12}\) coexist, and impaired vitamin B\(_{12}\) may negatively influence folate status,\(^27\) suggesting a need to be cognizant of vitamin B\(_{12}\) status when designing and implementing folic acid–fortification programs. An updated systematic review of worldwide vitamin B\(_{12}\) status seems warranted as a secondary priority to assessing folate status.

**Acknowledgments**
L.B.B. was responsible for the conception, overall content, and integrity of this review. D.B.H. assisted with manuscript drafting and revision and screened final content. Both authors read and approved the final version of the manuscript. The authors acknowledge the invaluable contribution of
Christine Pfeiffer in reviewing the methods-related content of the paper.

This paper was developed in support of the technical consultation *Folate Status in Women and Neural Tube Defect Prevention*, convened by the Micronutrient Forum and supported through Nutrition International by a grant provided by the Bill & Melinda Gates Foundation. An earlier version of this manuscript was presented to members of the technical on April 12–13, 2017, held at the Nutrition International headquarters in Ottawa, Ontario, Canada. This paper is being published individually but will be consolidated with other manuscripts as a special issue of *Annals of the New York Academy of Sciences*, under the coordination of Homero Martinez and Aliki P. Weakland. The special issue is the responsibility of the editorial staff of *Annals of the New York Academy of Sciences*, who delegated to the coordinators preliminary supervision of both technical conformity to the publishing requirements of *Annals of the New York Academy of Sciences* and general oversight of the scientific merit of each article. The authors alone are responsible for the views expressed in this paper; they do not necessarily represent the views, decisions, or policies of the institutions with which they are affiliated or the decisions, policies, or views of the Micronutrient Forum. The opinions expressed in this publication are those of the authors and are not attributable to the sponsors, publisher, or editorial staff of *Annals of the New York Academy of Sciences*.

**Competing interests**

The authors declare no competing interests.

**References**

1. Word Health Organization. 2015. Guideline: optimal serum and red blood cell folate concentrations in women of reproductive age for prevention of neural tube defects. Geneva: World Health Organization.

2. Pfeiffer, C.M., M. Zhang, D.A. Lacher, et al. 2011. Comparison of serum and red blood cell folate microbiologic assays for national population surveys. *J. Nutr. 141*: 1402–1409.

3. Pfeiffer, C.M., M. Zhang & S. Jabbar. 2018. Framework for laboratory harmonization of folate measurements in low- and middle-income countries and regions. *Ann. N.Y. Acad. Sci. 1414*: 96–108.

4. World Health Organization. 2015. Serum and red blood cell folate concentrations for assessing folate status in populations. Vitamin and Mineral Nutrition Information System. Geneva: World Health Organization.

5. Bailey, L.B., P.J. Stover, H. McNulty, et al. 2015. Biomarkers of nutrition for development—folate review. *J. Nutr. 145*: 1636S–1680S.

6. Bailey, L. & M. Caudill. 2012. Folate. In *Present Knowledge in Nutrition*. 10th ed. J. Erdman, I. MacDonald & S. Zeisel, Eds.: 321–342. Hoboken, NJ: Wiley-Blackwell.

7. Hao, L., J. Ma, M.J. Stampfer, et al. 2003. Geographical, seasonal and gender differences in folate status among Chinese adults. *J. Nutr. 133*: 3630–3635.

8. Pfeiffer, C.M., J.P. Hughes, D.A. Lacher, et al. 2012. Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988–2010. *J. Nutr. 142*: 886–893.

9. Yang, Q., M.E. Cogswell, H.C. Hamner, et al. 2010. Folic acid source, usual intake, and folate and vitamin B-12 status in US adults: National Health and Nutrition Examination Survey (NHANES) 2003–2006. *Am. J. Clin. Nutr. 91*: 64–72.

10. Berry, R.J., Z. Li, J.D. Erickson, et al. 1999. Prevention of neural-tube defects with folic acid in China. China–U.S. Collaborative Project for Neural Tube Defect Prevention. *N. Engl. J. Med. 341*: 1485–1490.

11. Yeung, L., Q. Yang & R.J. Berry. 2008. Contributions of total daily intake of folic acid to serum folate concentrations. *JAMA 300*: 2486–2487.

12. Hertrampf, E., F. Cortés, J.D. Erickson, et al. 2003. Consumption of folic acid-fortified bread improves folate status in women of reproductive age in Chile. *J. Nutr. 133*: 3166–3169.

13. Hopkins, S.M., M.J. Gibney, A.P. Nugent, et al. 2015. Impact of voluntary fortification and supplement use on dietary intakes and biomarker status of folate and vitamin B-12 in Irish adults. *Am. J. Clin. Nutr. 101*: 1163–1172.

14. Caudill, M.A., A.C. Cruz, J.F. Gregory, et al. 1997. Folate status response to controlled folate intake in pregnant women. *J. Nutr. 127*: 2363–2370.

15. Hao, L., Q.H. Yang, Z. Li, et al. 2008. Folate status and homocysteine response to folic acid doses and withdrawal among young Chinese women in a large-scale randomized double-blind trial. *Am. J. Clin. Nutr. 88*: 448–457.

16. Tighe, P., M. Ward, H. McNulty, et al. 2011. A dose-finding trial of the effect of long-term folic acid intervention: implications for food fortification policy. *Am. J. Clin. Nutr. 93*: 11–18.

17. Duffy, M.E., L. Hoey, C.F. Hughes, et al. 2014. Biomarker responses to folic acid intervention in healthy adults: a meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr. 99*: 96–106.

18. Wright, A.J., M.J. King, C.A. Wolfe, et al. 2010. Comparison of (6 S)-5-methyltetrahydrofolic acid v. folic acid as the reference folate in longer-term human dietary intervention studies assessing the relative bioavailability of natural food folates: comparative changes in folate status following a 16-week placebo-controlled study in healthy adults. *Br. J. Nutr. 103*: 724–729.

19. Pfeiffer, C.M., M.R. Sternberg, H.C. Hamner, et al. 2016. Applying inappropriate cutoffs leads to misinterpretation of folate status in the US population. *Am. J. Clin. Nutr. 104*: 1607–1615.
20. Daly, L.E., P.N. Kirke, A. Molloy, et al. 1995. Folate levels and neural tube defects. Implications for prevention. JAMA 274: 1698–1702.
21. Crider, K.S., O. Devine, L. Hao, et al. 2014. Population red blood cell folate concentrations for prevention of neural tube defects: Bayesian model. BMJ 349: g4554.
22. Shane, B. 2010. Folate chemistry and metabolism. In Folate in Health and Disease. 2nd ed. L. Bailey, Ed.: 1–24. Boca Raton, FL: CRC Press, Taylor Francis Group.
23. Mason, J.B. 2003. Biomarkers of nutrient exposure and status in one-carbon (methyl) metabolism. J. Nutr. 133(Suppl. 3): 941S–947S.
24. Clifford, A.J., E.M. Noceti, A. Block-Joy, et al. 2005. Erythrocyte folate and its response to folic acid supplementation is assay dependent in women. J. Nutr. 135: 137–143.
25. Houghton, L.A., A.R. Gray, M.C. Rose, et al. 2018. Should vitamin B supplementation represent a strategy to control neural tube defects? Pediat. Rev. 39: 118–145.
26. Wu, A., I. Chantarin, G. Slavin & A.J. Levi. 1975. Folate deficiency in the alcoholic—its relationship to clinical and haematological abnormalities, liver disease and folate stores. Br. J. Haematol. 29: 469–478.
27. Rosenthal, J., N. Largaespada, L.B. Bailey, et al. 2017. Folate deficiency is prevalent in women of childbearing age in Belize and is negatively affected by coexisting vitamin B-12 deficiency: Belize National Micronutrient Survey 2011. J. Nutr. 147: 1183–1193.
28. Molloy, A.M., P.N. Kirke, J.F. Troendle, et al. 2009. Maternal vitamin B12 status and risk of neural tube defects in a population with high neural tube defect prevalence and no folic acid fortification. Pediatrics 123: 917–923.
29. Molloy, A.M. 2018. Should vitamin B12 status be considered in assessing risk of neural tube defects? Ann. N.Y. Acad. Sci. 1414: 109–125.
30. Clarke, R., D.A. Bennett, S. Parish, et al. 2012. Homocysteine and coronary heart disease: meta-analysis of MTHFR case-control studies, avoiding publication bias. PLoS Med. 9: e1001177.
31. Yang, Q.H., L.D. Botto, M. Gallagher, et al. 2008. Prevalence and effects of gene–gene and gene–nutrition interactions on serum folate and serum total homocysteine concentrations in the United States: findings from the third National Health and Nutrition Examination Survey DNA Bank. Am. J. Clin. Nutr. 88: 232–246.
32. Crider, K.S., J.H. Zhu, L. Hao, et al. 2001. MTHFR 677C→T genotype is associated with folate and homocysteine concentrations in a large, population-based, double-blind trial of folic acid supplementation. Am. J. Clin. Nutr. 93: 1365–1372.
33. Yan, L., L. Zhao, Y. Long, et al. 2012. Association of the maternal MTHFR C677T polymorphism with susceptibility to neural tube defects in offspring: evidence from 25 case–control studies. PLoS One 7: e41689.
34. Zhang, T., J. Lou, R. Zhong, et al. 2013. Genetic variants in the folate pathway and the risk of neural tube defects: a meta-analysis of the published literature. PLoS One 8: e59570.
35. Metz, J. 2007. Folic acid metabolism and malaria. Food Nutr. Bull. 28(4 Suppl.): S540–S549.
36. Nzila, A., J. Okombo & A.M. Molloy. 2014. Impact of folate supplementation on the efficacy of sulfadoxine/pyrimethamine in preventing malaria in pregnancy: the potential of 5-methyl-tetrahydrofolate. J. Antimicrob. Chemother. 69: 323–330.
37. Gregson, A. & C.V. Plowe. 2005. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol. Rev. 57: 117–145.
38. Oppenheimer, S.J. & P. Cashin. 1986. Serum and red cell folate levels associated with malarial parasitaemia. Trans. R. Soc. Trop. Med. Hyg. 80: 169–171.
39. Chango, A. & L. Abdennabi-Najar. 2011. Folate metabolism pathway and Plasmodium falciparum malaria infection in pregnancy. Nutr. Rev. 69: 34–40.
40. Mazumdar, M., M.O. Ibane Hasan, R. Hamid, et al. 2015. Arsenic is associated with reduced effect of folic acid in myelomeningocele prevention: a case control study in Bangladesh. Environ. Health 14: 34.
41. Kamynina, E., E.R. Lachenauer, A.C. DiRisio, et al. 2017. Arsenic trioxide targets MTHFD1 and SUMO-dependent nuclear de novo thymidylate biosynthesis. Proc. Natl. Acad. Sci. USA 114: E2319–E2326.
42. Howe, C.G., M.M. Niedzwiecki, M.N. Hall, et al. 2014. Folate and cobalamin modify associations between S-adenosylmethionine and methylated arsenic metabolites in arsenic-exposed Bangladeshi adults. J. Nutr. 144: 690–697.
43. Hall, M.N. & M.V. Gamble. 2012. Nutritional manipulation of one-carbon metabolism: effects on arsenic methylation and toxicity. J. Toxicol. 2012: 959307.
44. Pfeiffer, C.M., M.R. Sternberg, R.L. Schleicher & M.E. Rybak. 2012. Dietary supplement use and smoking are important correlates of biomarkers of water-soluble vitamin status after adjusting for sociodemographic and lifestyle variables in a representative sample of U.S. adults. J. Nutr. 143: 9575–9658.
45. Rosenthal, J., M.E. Reeve, N. Ramirez, et al. 2016. Red blood cell folate insufficiency among nonpregnant women of childbearing age in Guatemala 2009 to 2010: prevalence and predicted neural tube defects risk. Birth Defects Res. A Clin. Mol. Teratol. 106: 587–595.
46. Branum, A.M., R. Bailey & B.J. Singer. 2013. Dietary supplement use and folate status during pregnancy in the United States. J. Nutr. 143: 486–492.
47. Parker, S.E., C.T. Mai, M.A. Canfield, et al. 2010. Updated national birth prevalence estimates for selected birth defects in the United States, 2004–2006. Birth Defects Res. A Clin. Mol. Teratol. 88: 1008–1016.
48. Centers for Disease Control and Prevention. 2010. CDC Grand Rounds: additional opportunities to prevent neural tube defects with folic acid fortification. MMWR Morb. Mortal. Wkly. Rep. 59: 980–984.
49. MRC Vitamin Study Research Group. 1991. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. Lancet 338: 131–137.
50. Czeizel, A.E. & I. Dudás. 1992. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N. Engl. J. Med. 327: 1832–1835.
51. Cortés, E., C. Mellado, R.A. Pardo, et al. 2012. Wheat flour fortification with folic acid: changes in neural tube defects rates in Chile. Am. J. Med. Genet. A 158A: 1885–1890.
52. De Wals, P., F. Tairou, M.I. Van Allen, et al. 2007. Reduction in neural-tube defects after folic acid fortification in Canada. *N. Engl. J. Med.* **357**: 135–142.

53. De Wals, P., F. Tairou, M.I. Van Allen, et al. 2008. Spina bifida before and after folic acid fortification in Canada. *Birth Defects Res. A Clin. Mol. Teratol.* **82**: 622–626.

54. Chen, L.T. & M.A. Rivera. 2004. The Costa Rican experience: reduction of neural tube defects following food fortification programs. *Nutr. Rev.* **62**(6 Pt 2): S40–S43.

55. Williams, L.I., C.T. Mai, L.D. Edmonds, et al. 2002. Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. *Teratology* **66**: 33–39.

56. Sayed, A.R., D. Bourne, R. Pattinson, et al. 2008. Decline in the prevalence of neural tube defects following folic acid fortification and its cost–benefit in South Africa. *Birth Defects Res. A Clin. Mol. Teratol.* **82**: 211–216.

57. Center for Disease Control. 1992. Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. *MMWR Recomm. Rep.* **41**: 1–7.

58. Tinker, S.C., H.C. Hamner, Y.P. Qi & K.S. Crider. 2015. U.S. women of childbearing age who are at possible increased risk of a neural tube defect–affected pregnancy due to suboptimal red blood cell folate concentrations, National Health and Nutrition Examination Survey 2007 to 2012. *Birth Defects Res. A Clin. Mol. Teratol.* **103**: 517–526.

59. Molloy, A.M. & J.M. Scott. 1997. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. *Methods Enzymol.* **281**: 43–53.

60. Serdula, M.K., E.K. Nichols, N.J. Aburto, et al. 2014. Micronutrient status in Jordan: 2002 and 2010. *Eur. J. Clin. Nutr.* **68**: 1124–1128.

61. Labadarios, D., R. Swart, E. Maudner, et al. 2008. Executive summary of the National Food Consumption Survey Fortification Baseline (NFCS-FB-I). *S. Afr. J. Clin. Nutr.* **21**(Suppl. 2): 247–300.

62. Freire, W., M. Ramirez-Luzuriaga, P. Belmont, et al. 2014. Tomo I: Encuesta Nacional de Salud y Nutrición de la población ecuatoriana de cero a 59 años. ENSANUT-ECU 2012. Ministry of Public Health/National Institute of Statistics and Census, Quito.

63. Rosenthal, J., E. Lopez-Pazos, N.F. Dowling, et al. 2015. Folate and vitamin B12 deficiency among non-pregnant women of childbearing-age in Guatemala 2009–2010: prevalence and identification of vulnerable populations. *Matern. Child Health J.* **19**: 2272–2285.

64. Imhoff-Kunsch, B., R. Flores, O. Dary & R. Martorell. 2007. Wheat flour fortification is unlikely to benefit the neediest in Guatemala. *J. Nutr.* **137**: 1017–1022.

65. Garrett, G.S. & L.B. Bailey. 2018. A public health approach for preventing neural tube defects: folic acid fortification and beyond. *Ann. N.Y. Acad. Sci.* **1414**: 47–58.

66. McLean, E., B. de Benoist & L.H. Allen. 2008. Review of the magnitude of folate and vitamin B12 deficiencies worldwide. *Food Nutr. Bull.* **29**(Suppl. 2): S38–S51.

67. Institute of Medicine. 1998. DRI dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington, DC: National Academy Press.