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Approaches to improving micronutrient status assessment at the population level

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Optimising micronutrient status globally is a major health priority. Nutritional biomarkers are critical for the identification of nutrient inadequacies in light of the limitations of dietary assessment methods. Early diagnosis and prevention of nutrient inadequacies require sensitive, validated and harmonised methods to determine and monitor micronutrient status in individual healthcare and population-based surveys. Important criteria in the identification, validation and implementation of nutritional biomarkers include the testing of biomarker specificity and sensitivity, and their response to dietary as well as physiologic changes, e.g. age or pregnancy. Nutritional status can be categorised into deficient, suboptimal, adequate and excess status, where appropriate, and provided cut-offs are available. Cut-offs are quantitative measures to reflect health outcomes and are important in validating nutritional surveys, interventions and monitoring of populations. For many biomarkers, available cut-offs have limited interpretability and are most commonly derived in adult populations only. For the comparison of studies from across the globe, the harmonisation of analytical methods is essential and can be realised with the use of internationally available reference material and interlaboratory comparison studies. This narrative review describes current efforts on identifying and validating existing and new biomarkers, the derivation of biomarker cut-offs, and international efforts on harmonisation of laboratory methods for biomarker quantitation and their interpretation, in the example of B-vitamins. Establishing sensitive, reliable and cost-efficient biomarkers and related cut-offs for use in populations across the globe are critical to facilitating the early diagnosis of micronutrient inadequacies on the clinical and community-based level for timely intervention and disease prevention.

Micronutrients: Nutritional status: Vitamins: Biomarker

The global burden of disease is high from single and multiple micronutrient deficiencies that are estimated to affect over two billion people and at least half of all children aged 6 months to 5 years[1]. Micronutrient deficiencies impact growth and key developmental outcomes in early life, and susceptibility and exacerbation of disease and loss in potential across the lifecycle[1]. While micronutrient deficiencies are a global health issue leading to detrimental health consequences and long-term impairment, suboptimal, that is subclinical or marginal, micronutrient status has also been associated with adverse health outcomes.

Nutritional status assessment using biochemical indicators, i.e. biomarkers, measured in accessible tissue, e.g. blood or urine, is an important tool for the diagnosis and monitoring of micronutrient status in individual

Abbreviations: MMA, methylmalonic acid; NHANES, National Adult Health and Nutrition Survey; PLP, pyridoxal 5'-phosphate; tHcy, total homocysteine.

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healthcare and population-based surveys. Using reliable and valid biomarkers to determine the nutritional status of a population is crucial for disease prevention strategies[2]. To facilitate reliable and early diagnosis of inadequate nutritional status, prior to the development of physiologic symptoms or impaired health, it is critical to have reliable and sensitive biomarkers, convenient, cost-efficient and accessible assays, and validated and internationally harmonised laboratory methods.

In the example of B-vitamins, this narrative review will discuss challenges of existing biomarkers, recent research on the development and testing of new biomarkers, followed by the importance and need for biomarker cut-offs as well as the potential need for age, life stage and ethnic-specific reference intervals. The third part focuses on results of recent interlaboratory comparison studies for harmonisation of existing assays and ongoing international efforts on the development of state-of-the-evidence information material for the selection and interpretation of nutritional biomarkers.

Micronutrient inadequacies: addressing deficiency and suboptimal status

Classical clinical symptoms of micronutrient deficiencies are most known and clinical indicators are well-established for their diagnosis. However, not only chronic micronutrient deficiencies, but also suboptimal micronutrient status, also referred to as marginal or subclinical deficiencies, have been associated with an increased risk of degenerative diseases, as explained herein in the example of B-vitamins.

The B-vitamins folate, vitamin B_{12}, B_6 and B_2 (i.e. riboflavin) are crucial nutrients for lifelong health given their role in cell formation and a healthy nervous system[3]. Folate, vitamin B_{12}, B_6 and riboflavin, together with choline, betaine and methionine, are described as methyl nutrients and have inter-related roles in the C_1 metabolism[3]. The C_1 metabolism is critical for basic cellular function and is characterised by the transfer of methyl or other C_1 groups in the folate cycle, including for DNA biosynthesis and for DNA and RNA methylation, that are critical epigenetic mechanisms. Vitamin B_{12} also serves as a coenzyme in the degradation of branched-chain amino acids and odd-chain fatty acids[4]. In addition to the C_1 metabolism, vitamin B_6 and riboflavin also have interdependent roles in the tryptophan-catabolising kynurenine pathway, which forms immunomodulatory metabolites[5]. B_6, as pyridoxal 5'-phosphate (PLP), is a coenzyme for >160 reactions[5]. Riboflavin also functions in iron and energy metabolism[6].

Low levels of folate, vitamin B_{12} and B_6 have been related to the increased risk of chronic diseases such as CVD[7,8], stroke[9], cognitive impairment[10,11] and cancer[12,13]. Suboptimal B_6 and B_2 are linked with higher colorectal cancer risk[14,15]. Low B_6 status is associated with higher risk for breast[12] and lung cancer[16], coronary artery disease[17,18], stroke[19] and Alzheimer's disease[10]. Riboflavin status has also been inversely associated with elevated total homocysteine (tHcy)[20] and with blood pressure[21], a major CVD risk factor. Suboptimal folate and B_{12} status have also been associated with adverse pregnancy outcomes such as neural tube defects[22,23] which reflects the importance of these micronutrients across the lifecycle.

Depending on the nutrient and population, the prevalence of suboptimal micronutrient status may be higher than that of deficiency, as is the case for vitamin B_{12} status in Canadian adults aged >19 years with 5 % being categorised as B_{12} deficient and 19 % having suboptimal B_{12} status[24]. To derive reliable and valid measures of nutrient adequacy, or optimal nutrient status, that is in the absence of clinical signs of deficiency and with optimal nutrient levels allowing for risk reduction of chronic degenerative diseases, sensitive and specific indicators of micronutrient status are needed that respond predictably to changes in micronutrient intake and body stores.

Evaluation of established and new biomarkers

Sensitive biomarkers are critical for the early identification of nutrient inadequacies. Recent research has focused on identifying, characterising and validating early indicators of micronutrient inadequacy.

Biomarkers can be divided into two categories, which are direct and functional indicators. Direct indicators are circulating concentrations of the micronutrient under investigation. In the example of folate, the direct indicators include serum folate and erythrocyte folate concentrations. Serum or plasma folate concentration is a short-term indicator and is impacted by dietary folate intake, postprandial status and genetic modifiers[3]. Erythrocyte folate concentration is regarded as a long-term indicator of folate status because erythrocytes incorporate folate only during erythropoiesis, excrete folate during their degeneration and have a half-life of about 60 d[25]. While serum or plasma folate is sensitive to changes in folate intake, serum total vitamin B_{12}, for example, is considered a direct indicator of low specificity and sensitivity[26]. An alternative direct indicator for vitamin B_{12} status is serum holotranscobalamin, the B_{12} form available to tissues, that was described to be more sensitive to dietary changes compared with total B_{12} [27,28].

In contrast to direct indicators, functional indicators reflect metabolic or functional consequences of an inadequate micronutrient status and are referred to as indicator of intracellular micronutrient deficiency. In the example of vitamin B_{12} that serves as a coenzyme in the folate-dependent homocysteine remethylation pathway and in the conversion of methylmalonyl-CoA to succinyl-CoA, the two functional indicators of vitamin B_{12} status are an increased concentration of the substrates of these two vitamin B_{12}-dependent pathways, i.e. elevated plasma tHcy and elevated methylmalonic acid (MMA), a byproduct of methylmalonyl-CoA, respectively[26].

Depending on the kinetic of each micronutrient, and thereby how promptly and predictably the direct indicator responds to changes in nutrient intake and body pool size, the diagnosis of nutritional inadequacies may for some micronutrients be best assessed by functional indicators or a combination of both direct and functional

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indicators. In the case of vitamin B_{12}, the functional indicator plasma MMA is described as the B_{12} biomarker with the highest sensitivity and specificity compared with other B_{12} indicators; however, plasma MMA is influenced by kidney function[29] and genetic modifiers[30]. Therefore, an expert panel concluded that the assessment of nutritional B_{12} status should include the measurement of at least one direct, e.g. serum total B_{12}, and one functional indicator, preferably MMA[26], to compensate for individual biomarker limitations through the combined use and interpretation of indicators. In the example of riboflavin, the only recognised biomarker for assessing biochemical riboflavin status is the functional indicator erythrocyte glutathione reductase activation coefficient[31], given the current lack of a convenient and validated direct indicator. The measurement of erythrocyte glutathione reductase activation coefficient however requires specific and laborious pre-analytical procedures and there is a lack of global accessibility to this assay, resulting in little knowledge about biochemical riboflavin status worldwide.

In light of the findings that suboptimal B_6 and B_2 status are linked with top causes of morbidity and mortality in industrialised countries, i.e. CVD and cancer, recent research focused on the identification and validation of new and early indicators of functional B_6 deficiency. Plasma cystathionine concentration is a sensitive functional indicator of suboptimal B_6 status, as shown in healthy young adult men and women after a 28 d dietary B_6 restriction[32,33], and seems the most sensitive indicator related to intermediates of the C_1 metabolism[34]. The team of Per Ueland[35] developed novel analytical assays for quantitation of B_6 and B_2 vitamers and biomarkers related to B-vitamin-dependent pathways including the tryptophan–kynurenine pathway that requires both B_6 and B_2[36,37]. The newly defined biomarkers include the PAr index (i.e. the ratio between the concentration of the B_6 degradation product pyridoxic acid, and the sum of the direct indicators PLP and pyridoxal), the 3-hydroxykynurenine:xanthurenic acid ratio and the oxoglutarate:glutamate ratio[5]. As for vitamin B_{12}, the combined use of biomarkers is recommended to compensate for the influence of potential confounding variables on each biomarker[5]. When validating the new biomarkers, plasma 3-hydroxykynurenine was inversely associated with plasma PLP, the most commonly used direct indicator of B_6 status, in a healthy population[38] and in patients with stable angina pectoris[39]. Because this association was independent of riboflavin, plasma 3-hydroxykynurenine concentration may serve as a specific, functional indicator of vitamin B_6 status[38]. Prospective and dose–response intervention studies are needed to validate the new biomarkers and to test the effect of nutritional status on their dynamics.

Derivation of biomarker cut-offs

Nutritional status can be categorised into deficient, suboptimal, adequate and excess status, provided cut-offs are available. Cut-offs are quantitative measures derived to reflect health outcomes and are critical in the validation of nutritional surveys, interventions and population-based screenings and monitoring. The derivation of cut-offs for nutritional biomarkers was comprehensively reviewed by Raghavan *et al.*[2]. In brief, cut-offs can be derived using receiver operating characteristics curves, Youden index or the calculation of reference intervals[2,40,41]. Receiver operating characteristics curves have commonly been used to derive cut-offs for nutritional biomarkers and can be used to compare biomarker effectiveness in distinguishing nutritional inadequacy from adequacy[2]. Receiver operating characteristics curves allow the calculation of area-under-the-curve values that are a summary measure of the accuracy of the cut-off, with an increasing diagnostic ability of the biomarker with increasing area-under-the-curve values[2]. The Youden index is a measure of overall diagnostic effectiveness derived from the receiver operating characteristics curve and used to interpret and evaluate biomarkers[2,40]. Reference intervals are also commonly used to generate cut-offs[2] and are critical in hospital laboratory settings for derivation of instrument-specific reference ranges of clinical indicators. Reference intervals are derived from reference values, i.e. biomarker concentrations, determined in a reference population, i.e. a sample of the target population. The reference intervals are values in the central 95 % of the distribution and allow the estimation of the upper and lower reference limits, i.e. the upper 97·5 % and lower 2·5 % of biomarker concentrations in the reference population[42].

For many biomarkers, available cut-offs have limited interpretability and have most commonly been derived in adult populations only. In the example of vitamin B_{12}, the combined assessment of at least one direct and one functional indicators is recommended[26]. There is a lack of consensus on available cut-offs for B_{12} biomarkers. Limited interpretability of existing cut-offs for serum total B_{12} and MMA to categorise into deficient, suboptimal and adequate B_{12} status has been reviewed by Carmel[43]. With data from the US National Adult Health and Nutrition Survey (NHANES 1999–2004), Bailey *et al.*[44] showed that commonly used cut-offs for serum total B_{12} and plasma MMA lead to a substantial level of misclassifications, i.e. a large proportion of individuals with normal total B_{12} concentration had elevated MMA, and 2 % of individuals had normal MMA but low serum total B_{12} concentration. Current cut-offs need to be validated using defined physiologic and/or clinical endpoints. Until those are established, as Bailey *et al.* concluded, 'the public health burden of vitamin B_{12} deficiency cannot accurately be estimated'[44] in our populations.

Influencing factors on biomarker variability and cut-offs

Current research is addressing the need and derivation of population-specific reference intervals and cut-offs. While micronutrients are critical across the lifespan, physiologic changes related to age, sex or specific life stages, e.g. pregnancy, likely impact the variability of biomarker concentrations and thereby the interpretability of available

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cut-offs. The age-related variability of biomarker concentrations is reflected in the example of serum folate concentrations measured in the US population as part of the NHANES 2003–2006. Serum folate concentrations were shown to decrease by mid-age and increase again with increasing age, also described as a U-shaped behaviour across the lifespan, that is independent of dietary folate intake[45].

The paediatric population demonstrates rapid growth and development, including continuous changes in metabolism, making reliable age-specific cut-offs necessary for accurate screening of nutritional inadequacies. To address the lack of paediatric reference ranges, the Canadian Laboratory Initiative for Pediatric Reference Intervals project was created with the goal to establish a database of reference intervals for biochemical indicators in children and to describe the influence of ethnicity, age, sex and BMI on biomarker concentrations[46]. To date, the Canadian Laboratory Initiative for Pediatric Reference Intervals project included the collection of blood samples and data from over 9000 healthy children aged 0–18 years, and reference intervals have been derived for over 100 medical tests and biomarkers, including for serum total vitamin B_{12}[47].

Sex-specific differences in biomarker concentrations and kinetics may apply, and sex should be evaluated as potential partition criterion in the derivation of cut-offs. Male–female differences were reported for plasma PLP concentration across the lifespan, in the NHANES 2003–2004[48]. Also circulating concentrations of functional indicators of B_6 status, specifically metabolites of the tryptophan–kynurenine pathway, greatly differed between young adult men and women[49]. The differences in biomarker variability between males and females, those related to biological differences, can be explained by sex-specific or sex-related physiologic differences, e.g. hormonal, and distinct characteristics in body composition, as well as possible sex-linked genetic differences. Sex-specific cut-offs may be less applicable in infants and children, until the age of puberty; sex-specific reference intervals were described for plasma tHcy with higher concentrations in boys compared with girls aged older than 12 years[47].

In regard to life stages, there is a lack of pregnancy-specific biomarker cut-offs for vitamin B_{12} and most other nutrients, which challenges the interpretation of micronutrient status assessment during this critical period of life and key stages of fetal development. Low maternal vitamin B_{12} status is a risk factor for neural tube defects[23], low birth weight[50] and reduced cognitive performance in the offspring[51]. With respect to the routinely applied biomarker serum total vitamin B_{12} and related cut-off, there is a general concern that the rate of B_{12} deficiency is overestimated during pregnancy because of a natural decrease in serum total B_{12} concentrations across trimesters of pregnancy[52–54], potentially attributable to pregnancy-related haemodilution, changes in glomerular filtration rate, and the preferential unidirectional transport of nutrients to the developing fetus. Also serum holotranscobalamin, the biomarker reflecting the portion of vitamin B_{12} available to tissues and alternative direct B_{12} indicator, significantly decreased between first and second trimester of pregnancy[53], while the functional biomarker plasma MMA increased between the first and second[53], and between second and third trimester[55]. As for cut-offs for all other age and population groups, a defined clinical and/or physiologic endpoint is required to validate cut-offs for the reliable definition of maternal vitamin B_{12} adequacy and thereby for achieving both maternal health and optimal fetal growth and development.

Consideration of ethnicity as an effect modifier in biomarker concentration and potential risk marker should be carefully evaluated. In our work on maternal micronutrient status in pregnant women of South Asian ethnicity residing in British Columbia, Canada, we showed that women of South Asian ethnicity have substantially lower vitamin B_{12} status, as shown by the significantly lower serum total B_{12} and holotranscobalamin concentration and higher serum MMA concentration, compared with women of European ethnicity in early pregnancy[53]. There was no significant difference in tHcy concentration between the two ethnic groups; however, this can likely be explained by the high folate status in Canadian pregnant women including our cohort with a median serum folate concentration of about 65 nmol/l[53] and with folate being the main determinant of tHcy concentration[56]. In late pregnancy, i.e. in second or third trimester, pregnant women of South Asian ethnicity had the highest risk of B_{12} deficiency compared with women of other ethnicities[57]. Underlying causes may be differences in dietary B_{12} intake and supplement use related to cultural habits and traditions, potentially higher prevalence of vegetarianism, and/or higher prevalence of genetic variants related to lower serum total B_{12} concentrations[58]. However, in a cross-sectional study of young adult women of South Asian and European ethnicity, dietary B_{12} intake did not differ between ethnic groups despite a trend for lower B_{12} status in South Asian young adult women[59].

Other influencing factors of biomarker concentrations include nutrient–nutrient interactions, drug–nutrient interactions and genetic variants. In the example of B_6, oral contraceptive use is associated with lower plasma PLP concentration likely because oestrogen enhances B_6-dependent tryptophan catabolism[60]. Inflammatory conditions are associated with low B_6 status[17]; and high dietary B_6 intake is associated with lower levels of inflammatory markers[60]. Genetic variants of proteins involved in B_6 absorption, metabolism and excretion may lead to hypo- or hyper-responsiveness to dietary and/or supplemental intake. The variability of biomarker concentrations in light of these confounding factors requires further investigation in large-scale, population-based studies.

Current international efforts on harmonisation of biomarker measurements

For the comparison of studies from across the globe, the harmonisation of analytical methods is essential and can be realised with the use of internationally available reference material and interlaboratory comparison studies. The requirement for international harmonisation was

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showcased by the comparison of folate status between the US and Canadian adult population[61]. Erythrocyte folate concentration, the long-term indicator of folate status, was measured in the national population-based surveys in the USA and Canada, which are the US NHANES and the Canadian Health Measures Survey, respectively. Assays employed were the microbiological folate assay for the NHANES and an automated immunoassay in the Canadian survey[61]. Since implementation of mandatory food fortification with folic acid in North America, the US and Canadian populations are folate replete, and often very high folate concentrations are being reported[62], especially in supplement users[63]. Immunoassays have been developed for clinical laboratory settings and the diagnosis of folate deficiency, and have the limitation of a lack of linearity and poor assay performance at high folate concentrations 45,[64]. Despite recommended dilution steps, the assay accuracy decreases with increasing folate concentrations. To account for the different analytical methods used in the two surveys, a conversion equation was developed. With or without the adjustment of folate concentrations employing the conversion factor, the erythrocyte folate concentration in the Canadian survey were higher or lower compared with the US counterparts which led the authors to conclude that 'caution must be exercised in evaluating erythrocyte folate data from different countries because analytical methods are not readily comparable'[61].

International efforts have been made for the harmonisation of existing assays for several micronutrient biomarkers. Recent interlaboratory comparison studies for folate quantitation assays[65,66], led by Christine Pfeiffer's team at the US Centers for Disease Control and Prevention, showed good comparability between isotope-dilution liquid chromatography–tandem MS assays for the quantitation of the folate form 5-methyltetrahydrofolate, the main transport form of folate in plasma[65]. However, the results of the different laboratories using either the liquid chromatography–tandem MS method as initially developed by Christine Pfeiffer's team[67], or independently developed assays, showed low comparability in the quantitation of folic acid[65], the fully oxidised folate form commonly found in supplements and fortified foods, that occurs unmetabolised in plasma at intake levels >200 μg of a single dose[68]. Individual, national and international efforts should be made to enhance interlaboratory comparison studies and facilitate the harmonisation of biomarker measurements between laboratories. Affordable, accessible and certified reference materials for the various folate forms, and other micronutrient biomarkers, are needed to improve method accuracy[65] in individual laboratories and globally.

Also, there are ongoing international efforts on the development of state-of-the-evidence information material for the selection and interpretation of nutritional biomarkers. These efforts include the Biomarkers of Nutrition for Development expert panel reviews, of which the review on folate was recently published[45]. A project addressing the interpretability of biomarker concentration, specifically in settings of inflammation and malaria infection, is the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia project[69]. The outcomes of these efforts will inform guidelines and harmonise strategies in the diagnosis of micronutrient deficiencies.

Conclusions

Micronutrient adequacy is critical for optimal growth and development, and maintenance of health and potential. Establishing sensitive, reliable and cost-efficient biomarkers and related cut-offs for use in populations across the globe are critical to facilitating the early diagnosis of micronutrient inadequacies on the clinical and community-based level for timely intervention and disease prevention. International efforts should be continued, supported and enhanced to facilitate the creation of accessible and harmonised measures to diagnosing and monitoring micronutrient deficiencies. More research is warranted on the identification of nutrient–nutrient interactions, the influence of genetic variants, age, life stage, ethnicity and other potential factors on biomarker variability and related cut-offs. The accessibility to cost-efficient and validated assays should be a global health priority.

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Conflict of Interest

None.

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