

# Genetic Hemoglobin Disorders Rather Than Iron Deficiency Are a Major Predictor of Hemoglobin Concentration in Women of Reproductive Age in Rural Prey Veng, Cambodia<sup>1,2</sup>

Crystal D Karakochuk,<sup>3,6</sup> Kyla C Whitfield,<sup>3,6</sup> Susan I Barr,<sup>3</sup> Yvonne Lamers,<sup>3,6</sup> Angela M Devlin,<sup>4-6</sup> Suzanne M Vercauteren,<sup>4,6,7</sup> Hou Kroeun,<sup>8</sup> Aminuzzaman Talukder,<sup>8</sup> Judy McLean,<sup>3</sup> and Timothy J Green<sup>3,6\*</sup>

<sup>3</sup>Food, Nutrition, and Health, Departments of <sup>4</sup>Pathology and Laboratory Medicine, and <sup>5</sup>Pediatrics, University of British Columbia, Vancouver, Canada; <sup>6</sup>The Child and Family Research Institute, Vancouver, Canada; <sup>7</sup>Division of Hematopathology, Children and Women's Health Centre of British Columbia, Vancouver, Canada; and <sup>8</sup>Helen Keller International, Cambodia Country Office, Phnom Penh, Cambodia

## Abstract

**Background:** Anemia is common in Cambodian women. Potential causes include micronutrient deficiencies, genetic hemoglobin disorders, inflammation, and disease.

**Objectives:** We aimed to investigate factors associated with anemia (low hemoglobin concentration) in rural Cambodian women (18–45 y) and to investigate the relations between hemoglobin disorders and other iron biomarkers.

**Methods:** Blood samples were obtained from 450 women. A complete blood count was conducted, and serum and plasma were analyzed for ferritin, soluble transferrin receptor (sTfR), folate, vitamin B-12, retinol binding protein (RBP), C-reactive protein (CRP), and  $\alpha$ 1 acid glycoprotein (AGP). Hemoglobin electrophoresis and multiplex polymerase chain reaction were used to determine the prevalence and type of genetic hemoglobin disorders.

**Results:** Overall, 54% of women had a genetic hemoglobin disorder, which included 25 different genotypes (most commonly, hemoglobin E variants and  $\alpha$ <sup>3-7</sup>-thalassemia). Of the 420 nonpregnant women, 29.5% had anemia (hemoglobin <120 g/L), 2% had depleted iron stores (ferritin <15  $\mu$ g/L), 19% had tissue iron deficiency (sTfR >8.3 mg/L), <3% had folate deficiency (<3  $\mu$ g/L), and 1% had vitamin B-12 deficiency (<150 pmol/L). Prevalences of iron deficiency anemia (IDA) were 14.2% and 1.5% in those with and without hemoglobin disorders, respectively. There was no biochemical evidence of vitamin A deficiency (RBP <0.7  $\mu$ mol/L). Acute and chronic inflammation were prevalent among 8% (CRP >5 mg/L) and 26% (AGP >1 g/L) of nonpregnant women, respectively. By using an adjusted linear regression model, the strongest predictors of hemoglobin concentration were hemoglobin E homozygous disorder and pregnancy status. Other predictors were 2 other heterozygous traits (hemoglobin E and Constant Spring), parity, RBP, log ferritin, and vitamin B-12.

**Conclusions:** Multiple biomarkers for anemia and iron deficiency were significantly influenced by the presence of hemoglobin disorders, hence reducing their diagnostic sensitivity. Further investigation of the unexpectedly low prevalence of IDA in Cambodian women is warranted. *J Nutr* 2015;145:134–42.

**Keywords:** anemia, Cambodia, ferritin, hemoglobin, iron deficiency, micronutrient, serum transferrin receptor, thalassemia, women

## Introduction

Anemia is a severe public health problem for Cambodian women of reproductive age, affecting ~44% of the population (1–3).

Anemia negatively affects pregnancy outcomes (4–6) in addition to impairing work capacity and productivity of the women (7). Anemia is defined as a hemoglobin concentration <120 g/L in nonpregnant women of reproductive age and hemoglobin <110 g/L in pregnant women of reproductive age (1). The causes of anemia are multifactorial and can include factors related to nutrition (e.g., micronutrient deficiencies) (8–10), genetic hemoglobin disorders (e.g., thalassemia) (11, 12), as well as inflammation and disease (13–16).

<sup>1</sup> Supported by the International Development Research Centre; the Department of Foreign Affairs, Trade, and Development, Canada; and the Canadian Institutes of Health Research Vanier Graduate Scholarships.

<sup>2</sup> Author disclosures: CD Karakochuk, KC Whitfield, SI Barr, Y Lamers, AM Devlin, SM Vercauteren, H Kroeun, A Talukder, J McLean, and TJ Green, no conflicts of interest.

\* To whom correspondence should be addressed. E-mail: tgreen@mail.ubc.ca.

Iron deficiency is often assumed to be a major cause of anemia in Cambodia because of iron-poor diets that consist mainly of rice and lack iron-rich animal food sources (17). Also, impaired absorption and loss of iron can result from infection and disease (15, 18, 19) such as dengue, malaria, hookworm, and parasites, which are prevalent in Cambodia (20–22). Furthermore, in women of reproductive age, menstruation and pregnancy increase daily iron requirements (23–25), which, if not met, can contribute to anemia.

The accurate diagnosis of iron deficiency anemia (IDA)<sup>9</sup> in the developing world is challenging. Diagnostic criteria for women of reproductive age include hemoglobin concentration <120 g/L in conjunction with serum ferritin <15 µg/L or soluble transferrin receptor (sTfR) >8.3 mg/L (1, 18). However, serum ferritin becomes elevated in the presence of inflammation, limiting its diagnostic sensitivity (1, 26, 27). It is recommended to correct ferritin values for inflammation by using 2 inflammation biomarkers: C-reactive protein (CRP) and/or α1 acid glycoprotein (AGP) (1, 28). Alternatively, sTfR, an indicator of tissue iron deficiency, is less influenced by inflammation (29, 30) and has been suggested as a more sensitive indicator of iron deficiency in populations with a high prevalence of inflammation and/or disease (31).

However, not all anemias are caused by iron deficiency, and when iron deficiency is not a major cause, iron interventions such as fortification and/or supplementation are not effective to reduce or prevent anemia. Other micronutrient deficiencies including vitamin B-12, folate, vitamin A, and riboflavin can also cause anemia (9, 32–36). In addition, vitamin C (37, 38) and vitamin B-6 (39, 40) have been implicated in the development of anemia but have been less commonly examined.

In Cambodia, genetic hemoglobin disorders affect >50% of the total population. The most common disorders include hemoglobin E variants and α-thalassemias (41). These disorders are autosomal recessive, so can be inherited in either the homozygous form or the heterozygous form (which is also referred to as a “trait”). Hemoglobin disorders can result in decreased or defective hemoglobin production, leading to an increased risk of anemia and other serious health problems (11, 12). There are 2 main categories of genetic hemoglobin disorders: structural hemoglobin variants and thalassemias. Structural hemoglobin variants result from a single amino acid substitution in 1 of the globin chains of hemoglobin. Thalassemias result from a reduced synthesis of either the α- or β-globin chains of hemoglobin (referred to as α- or β-thalassemia, respectively) (11). Genetic hemoglobin disorders result in a range of outcomes, from asymptomatic conditions to severe anemia that is incompatible with life (i.e., hydrops fetalis). Both serum ferritin and sTfR concentrations were reported to be elevated in individuals with genetic hemoglobin disorders (42–46). This limits the diagnostic sensitivity of serum ferritin and sTfR to determine iron deficiency in individuals with hemoglobin disorders. However, the impact that genetic hemoglobin disorders have on anemia in rural Cambodian women is not known.

The aims of this cross-sectional survey are to investigate the factors associated with low hemoglobin concentration (anemia) in women of reproductive age (18–45 y) in rural Cambodia and

to investigate the relations between genetic hemoglobin disorders and the common biomarkers of iron deficiency (serum ferritin and sTfR).

## Methods

**Study design.** This cross-sectional study was conducted in women of reproductive age (18–45 y) in rural Prey Veng province in Cambodia. Sociodemographic and health data, anthropometric measurements, and a venous blood sample were collected from women in July 2012. Ethical approval for the study was granted by the Clinical Research Ethics Board at the University of British Columbia (Canada) and the National Ethics Committee for Health Research (Cambodia). Written informed consent was obtained from all women by ink-stamped thumbprint upon enrollment in the study.

**Recruitment and eligibility.** This cross-sectional analysis used baseline data that were obtained from 450 women in 4 districts (Mesang, Kamchay Mear, Svay Anthor, and Bar Phnom) of Prey Veng province for inclusion in a larger randomized controlled trial (TJ Green, unpublished data, 2014). The data were collected before the implementation of a trial designed to evaluate an improved model of homestead food production and aquaculture.

Women were recruited by using a 2-stage stratified cluster design. In consultation with provincial government officials and the Ministry of Planning, a list of all villages in the province was obtained. Villages ( $n = 6$ ) were systematically excluded if they were participating in other development programs, hence receiving products or interventions that could have biased outcomes in the larger trial. In the first stage, 90 villages were sequentially selected from a randomly ordered list of all eligible villages in the 4 districts. Within each selected village, households were sequentially selected from a randomly ordered list of all eligible households and visited to determine if they met the eligibility criteria. Selection from the list continued until 10 eligible households were recruited. To be eligible for inclusion the woman had to be between 18 and 45 y of age, have at least 1 child <5 y of age, and live in farming households with some access to land for agriculture or aquaculture activities. Last, 450 households were randomly selected, from which women provided blood samples.

**Sociodemographic and health data collection.** Data were collected in the local language by trained Cambodian research staff using interviewer-administered questionnaires that included the women's age, educational level, occupation, household size, parity, and pregnancy status. Health status indicators included the use of micronutrient or other supplements, medications, and disease state. All data were obtained by self-report from women at the household.

**Anthropometric measurements.** Weight and height were measured for each woman at the household by trained research staff using standardized techniques (47) and calibrated equipment. Duplicate measurements were taken, and the average value of the 2 measurements was used. BMI was calculated on the basis of weight and height measurements [weight (kg) divided by height (m<sup>2</sup>)].

**Blood collection, processing, and assessment.** A 3-h fasting venous blood sample was collected in the morning by trained phlebotomists from the Cambodian National Institute of Public Health Laboratory (NIPHL) at health centers in Prey Veng province. Blood was collected in 2 evacuated 3.5-mL tubes (Becton Dickinson), 1 of which contained an anticoagulant (EDTA). Samples were placed on ice and transported daily to NIPHL in Phnom Penh for processing. After processing, serum samples were placed in aliquots in multiple vials and stored at –70°C until shipment to the appropriate laboratories for analysis.

A complete blood count was performed by using an automated hematology analyzer (Sysmex XT-1800i; Sysmex Corporation) at NIPHL in Cambodia. Serum was analyzed for ferritin, sTfR, retinol binding protein (RBP), AGP, and CRP by using a sandwich ELISA (48) at the Erhardt Laboratory in Germany. Serum folate was analyzed by using

<sup>9</sup> Abbreviations used: AA, normal hemoglobin; AGP, α1 acid glycoprotein; CRP, C-reactive protein; CS, Constant Spring; IDA, iron deficiency anemia; MCV, mean corpuscular volume; NIPHL, National Institute of Public Health Laboratory; PLP, pyridoxal-5'-phosphate; RBP, retinol binding protein; sTfR, soluble transferrin receptor.

a 96-well plate microbiological assay (49), and serum vitamin B-12 was analyzed by using an autoanalyzer with appropriate controls (Abbott AxSym). Plasma pyridoxal-5'-phosphate (PLP; as an indicator of vitamin B-6 deficiency) was measured in a random subsample of 99 women by using HPLC according to the method by Ubbink et al. (50), with modifications, at the Lamers Laboratory at The University of British Columbia in Canada.

Genetic hemoglobin disorders were identified by using methods of hemoglobin electrophoresis and PCR (51). Capillary hemoglobin electrophoresis was conducted by using a Sebia MINICAP analyzer (hemoglobin E program) by a trained external consultant at NIPHL in Cambodia. This automated technique quantifies the different types of hemoglobin in blood for interpretive diagnosis. It can detect normal hemoglobin (A, A<sub>2</sub>, and F), hemoglobin variants [E, H, and Constant Spring (CS)], and  $\beta$ -thalassemia. The Sebia analyzer has an advantage over other methodology because it can distinguish between hemoglobin A<sub>2</sub> and hemoglobin E, both of which are common in Cambodia (52). Genomic DNA was extracted from buffy coat by using the QiaAmp Blood DNA kit, and a multiplex PCR assay (53) was used to detect heterozygosity, homozygosity, and compound heterozygosity of the 7 most common  $\alpha$ -globin gene deletions in cases of  $\alpha$ -thalassemia ( $\alpha^{SEA}$ ,  $\alpha^{20.5}$ ,  $\alpha^{MED}$ ,  $\alpha^{FIL}$ ,  $\alpha^{THAI}$ ,  $\alpha^{3.7}$ , and  $\alpha^{4.2}$ ) at the Molecular Genetics Laboratory at BC Children's Hospital in Canada.

Anemia was defined as hemoglobin <120 g/L for nonpregnant women and hemoglobin <110 g/L for pregnant women (1). Microcytic anemia was defined as hemoglobin <120 g/L and mean corpuscular volume (MCV) <80 fL. Depleted iron stores was defined as ferritin <15  $\mu$ g/L, and tissue iron deficiency was defined as sTfR >8.3 mg/L. IDA was defined as hemoglobin <120 g/L and either ferritin <15  $\mu$ g/L or sTfR >8.3 mg/L. Acute and chronic inflammation were defined as CRP >5 mg/L and AGP >1 g/L, respectively. The thresholds for defining deficiencies of other micronutrients were as follows: serum vitamin B-12 <150 pmol/L, serum folate <3  $\mu$ g/L, vitamin A as indicated by serum RBP <0.7  $\mu$ mol/L, and vitamin B-6 as indicated by plasma PLP <20 nmol/L.

**Statistical analysis.** Ferritin and RBP values were corrected for inflammation by using AGP and CRP biomarkers in accordance with published methods by Thurnham et al. (28, 54). On the basis of subclinical inflammation stages of incubation (elevated CRP), early convalescence (elevated CRP and AGP), and late convalescence (elevated AGP) among women, correction factors were applied to ferritin (0.77, 0.53, and 0.75) and RBP (1.13, 1.24, and 1.11) concentrations, respectively (28, 54). Ferritin, sTfR, folate, CRP, and AGP were not normally distributed in the population based on Shapiro-Wilks tests of normality ( $P < 0.05$ ) and were therefore natural ln-transformed before statistical analyses. The K-nearest neighbors imputation method was used (55) to generate data for women with missing vitamin B-12 values ( $n = 24$ ) due to incomplete laboratory analyses. The K-nearest neighbors imputation method input the median values of the 10 most similar samples in the data set to the samples with missing values. No other values were missing from the data set.

A predictive model of linear regression analyses was used to measure the association between hemoglobin concentration (continuous variable, g/L) and multiple independent variables. We included variables in the model that either had a bivariate linear relation ( $r > 0.2$ ) or that were commonly known to be associated with anemia (i.e., inflammation markers AGP and CRP), even if there was a weak bivariate relation detected in our data. Log ferritin, RBP, log sTfR, vitamin B-12, log folate, log AGP, log CRP, age, parity, and BMI were analyzed as continuous variables in the regression model. The 5 most common genetic hemoglobin disorders detected in the women were included in the model as nominal variables (coded as 0 or 1 according to whether they had or did not have the genetic hemoglobin disorder). Unstandardized  $\beta$  estimates with 95% CIs and standardized  $\beta$  estimates ( $\beta$ ) were used to describe the magnitude of associations.  $\beta$  Estimates result from separate analyses on standardized independent variables so that each variable has a variance of 1. ANOVA,  $t$  tests, and chi-square tests were used to assess baseline characteristics and conduct pairwise comparisons between groups (by using least significant difference to adjust for multiple comparisons where required). Two-sided  $P$  values <0.05

indicated significance. IBM SPSS software version 22 was used to conduct statistical analyses.

The sample size for this cross-sectional survey was estimated by using an online calculator tool for multiple regression models (56) and in consultation with a biostatistician. With the use of a 2-sided  $P$  value = 0.05, 80% power, 8 independent variables, and an expected multiple regression coefficient of  $R^2 = 0.2$ , the ideal sample size for this cross-sectional study was 367 women. Because blood samples and ethical consent were obtained from 450 women as the baseline survey of the larger trial (TJ Green, unpublished data, 2014), data from all 450 women were included in the statistical analysis.

## Results

**Demographic, health, and nutrition characteristics.** A total of 420 nonpregnant women and 30 pregnant women aged 18–45 y were recruited for the study (Table 1). Mean age, parity, and the prevalence of micronutrient deficiencies, inflammation, and anemia were reported for all women. BMI was reported only for nonpregnant women. Vitamin B-6 deficiency (as indicated by plasma PLP <20 nmol/L) was detected in 2 women in a random subsample of 99 women (~2% deficiency prevalence). Of the nonpregnant women with anemia, 68.5% had mild anemia (hemoglobin 110–120 g/L), 31.5% had moderate anemia (hemoglobin 80–110 g/L), no women had severe anemia (hemoglobin <80 g/L), and 62.9% had microcytic anemia (hemoglobin <120 g/L and MCV <80 fL). Of the pregnant women with anemia, 76.9% had mild anemia (hemoglobin 100–110 g/L), 23.1% had moderate anemia (hemoglobin 70–100 g/L), no women had severe anemia (hemoglobin <70 g/L), and 61.5% had microcytic anemia (hemoglobin <110 g/L and MCV <80 fL).

**Typing of genetic hemoglobin disorders.** Using capillary hemoglobin electrophoresis and multiplex PCR, we determined the frequency of all hemoglobin types detected in the 450 Cambodian women in the study (Table 2). Overall, 54% of women in the study had a genetic hemoglobin disorder. Twenty-six different genotypes were identified, including normal hemoglobin and single or coinherited variants and/or deletions. The most common abnormal genotypes included hemoglobin E trait, affecting 14.9% of women, and  $\alpha^{3.7}$ -thalassemia trait, affecting 11.6% of women. “Hemoglobin E homozygous” in Table 2 ( $n = 4$ ) refers only to women with independent hemoglobin E homozygous disorders. “Hemoglobin E homozygous group” in Tables 3 and 4 ( $n = 31$ ) refers to a group of women with any form of hemoglobin E homozygous disorders, including forms coinherited with other compound and heterozygous traits.

**Multivariate linear regression.** Using a multivariate linear regression model, we determined the predictors of hemoglobin concentration in the 450 Cambodian women (Table 3). Variables were added to the model in a forward stepwise conditional approach. The largest change in  $R^2$  occurred with the addition of the hemoglobin E homozygous group variable (13.4%) and the second largest change with pregnancy status (9.7%). Hence, hemoglobin E homozygous disorder and being pregnant were the strongest negative predictors in the model. The hemoglobin E homozygous group ( $n = 31$ ) included women with any hemoglobin E homozygous form, including forms coinherited with other compound and heterozygous traits ( $\alpha^{3.7}$ -thalassemia trait,  $\alpha^{SEA}$ -thalassemia trait, etc.). PLP (vitamin B-6) was not included in the model because we only collected data from a subsample of 99 women and deficiency prevalence was low

**TABLE 1** Demographic, health, and nutrition characteristics of 450 Cambodian women aged 18–45 y in rural Prey Veng province<sup>1</sup>

	Nonpregnant	Pregnant
Total, <i>n</i> (%)	420 (93.3)	30 (6.7)
Age, y	29.6 ± 6.5 <sup>2</sup>	28.5 ± 4.6
Micronutrient deficiencies, <i>n</i> (%)		
Vitamin B-12 <150 pmol/L	4 (1.0)	3 (10.0)
RBP (vitamin A) <sup>3</sup> <0.7 μmol/L	0 (0)	0 (0)
Folate <3 μg/L	11 (2.6)	0 (0)
Depleted iron stores (ferritin) <sup>3</sup> <15 μg/L	9 (2.1)	4 (13.3)
Tissue iron deficiency (sTfR) >8.3 mg/L	79 (18.8)	5 (16.7)
Inflammation, <i>n</i> (%)		
Acute, CRP >5 mg/L	35 (8.3)	5 (16.7)
Chronic, AGP >1 g/L	107 (25.5)	2 (6.7)
Parity (number of children ever born), <i>n</i> (%)		
1	132 (31.4)	18 (60.0)
2–3	211 (50.2)	10 (33.3)
≥4	77 (18.3)	2 (6.7)
BMI (kg/m <sup>2</sup> ), <i>n</i> (%)		
Underweight: <18.5	62 (14.8)	N/A
Normal: 18.5–24.9	318 (75.7)	N/A
Overweight: 25–29.9	33 (7.9)	N/A
Obese: ≥30	7 (1.7)	N/A
Anemia, <sup>4</sup> <i>n</i> (%)	124 (29.5)	13 (43.3)

<sup>1</sup> AGP, α1 acid glycoprotein; CRP, C-reactive protein; N/A, not applicable; RBP, retinol binding protein; sTfR, soluble transferrin receptor.

<sup>2</sup> Mean ± SD (all such values).

<sup>3</sup> Values were corrected for subclinical inflammation by using AGP and CRP biomarkers (28, 54).

<sup>4</sup> Hemoglobin <120 g/L (nonpregnant) and <110 g/L (pregnant).

(2%). Furthermore, a bivariate correlation showed no association between hemoglobin concentration and PLP (Pearson's  $r = 0.02$ ).

Other variables negatively associated with hemoglobin concentration in the model included parity (number of children previously born) and 2 other genetic hemoglobin disorders: hemoglobin E trait and hemoglobin CS trait. Other variables positively associated with hemoglobin concentration in the model included log ferritin (adjusted for inflammation), RBP, and vitamin B-12. However, these other variables had less magnitude than hemoglobin E homozygous disorder and pregnancy status variables.

The adjusted  $R^2 = 0.31$  in the model indicated that ~31% of the variance in the outcomes could be explained by the model. A bivariate correlation matrix confirmed that no 2 variables in the model were related beyond an acceptable level. In addition, variance inflation factors were calculated and indicated no multicollinearity between variables included in the model. This suggests that there were no interactions between the independent variables, which might compromise the fit of the model. Variables that were not significant in the model and therefore not included in the model were age, BMI, log sTfR, log folate, log CRP, log AGP, and 2 other genetic hemoglobin disorders: α<sup>3.7</sup>-thalassemia trait and coinherited hemoglobin E trait and α<sup>3.7</sup>-thalassemia trait.

Ferritin was adjusted for levels of subclinical inflammation by using AGP and CRP biomarkers before inclusion in the model. This was because log CRP and log AGP were not significant in the model when including the unadjusted log ferritin variables. Replacing unadjusted ferritin in the model with adjusted ferritin did not change the fit of the model (no change in  $R^2$ ).

**Hematologic indicators and biomarkers of iron deficiency.** Anemia prevalence and hematologic indicators were investigated

in the 7 most commonly detected types in the nonpregnant women in the study (Table 4). Table 4 does not include data for rare hemoglobin variants affecting <7 women in each category; accordingly, data are presented only for the 389 women with normal hemoglobin (AA), hemoglobin E trait, α<sup>3.7</sup>-thalassemia trait, coinherited hemoglobin E trait and α<sup>3.7</sup>-thalassemia trait, hemoglobin E homozygous, hemoglobin CS trait, and α<sup>3.7</sup>-thalassemia homozygous.

In pairwise comparisons, significant differences ( $P < 0.05$ , least significant difference adjusted for multiple comparisons) were detected between hematologic variables in women with AA compared with women with the common genetic hemoglobin disorders. Most remarkable was the significant difference in mean hemoglobin concentration in women in the hemoglobin E homozygous group (mean ± SD = 109 ± 7.3 g/L) compared with women with AA (130 ± 8.9 g/L). Anemia was also most prevalent among the hemoglobin E homozygous group (including those with other coinherited compound and heterozygous traits, such as hemoglobin CS trait, α<sup>SEA</sup>-thalassemia trait, etc.): 30 of 31 women (97%) had anemia.

MCV and mean corpuscular hemoglobin concentrations were significantly lower among women with any of the 6 most common genetic hemoglobin disorders compared with women with AA. Low MCV and mean corpuscular hemoglobin concentrations are defined as microcytic and hypochromic anemia, respectively, and can be a consequence of IDA, genetic hemoglobin disorders, chronic disease, or other factors (12). Red blood cell distribution width was elevated in all 6 of the most common genetic hemoglobin disorders.

Ferritin concentrations were significantly higher in women in the hemoglobin E homozygous disorders group compared with women with normal hemoglobin. No other genetic hemoglobin



**TABLE 2** Frequency of all hemoglobin types detected in 450 Cambodian women aged 18–45 y in rural Prey Veng province<sup>1</sup>

	<i>n</i>	%
AA	207	46.0
Abnormal hemoglobin	243	54.0
Hemoglobin E trait	67	14.9
α <sup>3.7</sup> -Thalassemia trait	52	11.6
Hemoglobin E trait and α <sup>3.7</sup> -thalassemia trait	35	7.8
Hemoglobin E homozygous with increased hemoglobin F	22	4.9
Hemoglobin CS trait	15	3.3
α <sup>3.7</sup> -Thalassemia homozygous	8	1.8
Hemoglobin F increased in isolation	6	1.3
β <sup>+</sup> -Thalassemia trait <sup>2</sup>	4	0.9
Hemoglobin E homozygous	4	0.9
Hemoglobin E trait and α <sup>3.7</sup> -thalassemia homozygous	3	0.7
Hemoglobin E trait and hemoglobin CS trait	3	0.7
β <sup>+</sup> -Thalassemia trait <sup>2</sup> with increased hemoglobin F	3	0.7
Hemoglobin E trait with increased hemoglobin F	3	0.7
α <sup>SEA</sup> -Thalassemia trait	3	0.7
Hemoglobin E trait and α <sup>SEA</sup> -thalassemia trait	2	0.4
Hemoglobin E trait and α <sup>4.2</sup> -thalassemia trait	2	0.4
Hemoglobin CS trait and α <sup>3.7</sup> -thalassemia trait	2	0.4
Hemoglobin E homozygous and α <sup>3.7</sup> -thalassemia trait	2	0.4
Hemoglobin E trait and α <sup>SEA</sup> -thalassemia trait and α <sup>3.7</sup> -thalassemia trait	1	0.2
Hemoglobin E trait and hemoglobin CS trait and α <sup>3.7</sup> -thalassemia trait	1	0.2
β <sup>+</sup> -Thalassemia trait <sup>2</sup> and hemoglobin CS trait	1	0.2
Hemoglobin Bart and α <sup>3.7</sup> -thalassemia trait	1	0.2
Hemoglobin E homozygous and α <sup>3.7</sup> -thalassemia homozygous	1	0.2
Hemoglobin E homozygous and α <sup>4.2</sup> -thalassemia trait	1	0.2
Hemoglobin E homozygous and α <sup>SEA</sup> -thalassemia trait with increased hemoglobin F	1	0.2

<sup>1</sup> AA, normal hemoglobin; CS, Constant Spring; SEA, South East Asia.

<sup>2</sup> β<sup>+</sup>-Thalassemia trait is suspected on the basis of an elevated hemoglobin A<sub>2</sub> (>3.5%) and hematologic values (low hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin); however, elevated hemoglobin A<sub>2</sub> could also be due to other causes.

disorders statistically increased ferritin concentration. sTfR concentrations were significantly elevated in 3 of the 6 genetic hemoglobin disorders (hemoglobin E trait, hemoglobin E homozygous group, and hemoglobin CS trait). There were no significant differences in mean RBP, AGP, and CRP concentrations across different hemoglobin disorders.

The prevalence of iron deficiency with and without anemia among women with or without genetic hemoglobin disorders was also compared (Table 5). Overall, those women with any form of genetic hemoglobin disorder had an iron deficiency prevalence of ~27% compared with ~11% among women with no genetic hemoglobin disorder. Those women with any form of genetic hemoglobin disorder had an anemia prevalence of ~45% compared with ~11% among women with no genetic hemoglobin disorder ( $P < 0.05$ ). Last, those women with any form of genetic hemoglobin disorder had an IDA prevalence of ~14% compared with <2% among women with no genetic hemoglobin disorder ( $P < 0.05$ ). However, when using only ferritin cutoffs for IDA, the prevalence of IDA among women with no genetic hemoglobin disorder was only ~1%.

We also compared the proportion of women with low ferritin concentrations in nonpregnant women without genetic hemoglobin disorders between women with and without chronic inflammation but did not detect a difference between groups.

However, there was a significant difference using sTfR as a biomarker. A significantly higher proportion of women with chronic inflammation (17.5%) had an elevated sTfR concentration (>8.3 mg/L) than did women without chronic inflammation (7.2%) ( $P = 0.03$ ). Although it is currently thought that sTfR is not influenced by inflammation (1, 29), our data suggest otherwise.

## Discussion

The current study highlighted the complexity and diverse heterogeneity of hemoglobin disorders in Cambodia. Normal hemoglobin consists of α- and β-globin chains, encoded by 4 α genes and 2 β genes (11). The complexity of these disorders is a result of the many possible combinations of gene variations or deletions that can occur in single, compound, and/or coinherited forms in these 6 genes. The frequencies of genotypes detected in the women in our study are similar to other reports in Cambodian children (41, 44, 57). Of all the genetic hemoglobin disorders, hemoglobin E homozygous was the strongest (negative) predictor of hemoglobin concentration in our regression model.

George et al. (44) also found that hemoglobin E homozygous was the most significant predictor of hemoglobin among 2329 children in Cambodia. In contrast to our study, George et al. (44) also found that log sTfR, age, and AGP were significant (negative) predictors of hemoglobin. However, the study was conducted among children aged 6–59 mo, an age group known to be at high risk of iron deficiency, rather than women aged 18–45 y in our study. The α<sup>3.7</sup>-thalassemia trait and coinherited hemoglobin E trait and α<sup>3.7</sup>-thalassemia trait were not significant predictors and therefore not included in the model, despite our finding that women with these hemoglobin disorders had significantly lower hemoglobin concentrations compared with women with normal hemoglobin (Table 3). We speculate this could be due to the small sample size of some of the rare disorders. For similar reasons, we only included the 5 most common hemoglobin disorders in the regression model. However, there are rare hemoglobin disorders that likely would be strong predictors of hemoglobin. For example, β-thalassemia major (homozygosity or compound heterozygosity) is known to result in severe anemia (11); however, the frequency of this genotype in Cambodia is very low. No women with this genotype were detected in our study; therefore, it was not included in our model. However, it still remains important in consideration of its hematologic consequences and risk of anemia.

It is not surprising that ferritin, RBP, and vitamin B-12 were all positively associated with hemoglobin concentration. This is because of the potential role that these biomarkers and micronutrients play in the etiology of anemia (8, 10). We suspect these variables may have been stronger predictors in the model if indeed there were higher rates of deficiencies in this population. It is also not surprising that pregnancy and parity were negative predictors of hemoglobin in the model. Blood volume is increased substantially during pregnancy and results in a diluted hemoglobin concentration in women (12, 18). Furthermore, in the second and third trimesters of pregnancy, daily iron requirements increase (23, 24), which, in turn, can contribute to the risk of anemia. The demands of multiple, consecutive pregnancies can negatively influence maternal nutrition status (namely anemia), especially in closely spaced pregnancies in which there is limited time for recovery and iron stores repletion between births (58).

The most unexpected finding in this study among rural Cambodian women was the extremely low prevalence of IDA

**TABLE 3** Predictors of hemoglobin concentration (g/L) in 450 Cambodian women aged 18–45 y by using a multivariate linear regression model<sup>1</sup>

	Unstandardized coefficients		Standardized coefficient $\beta$	P
	B (95% CI)	SE		
Constant	108.09 (101.45, 114.73)	3.38	—	<0.0001
Genetic hemoglobin disorders				
Hemoglobin E homozygous group <sup>2</sup>	−18.24 (−21.74, −14.73)	1.78	−0.41	<0.0001
Hemoglobin E trait	−4.33 (−7.06, −1.59)	1.39	−0.12	0.002
Hemoglobin CS trait	−5.57 (−10.45, −0.70)	2.48	−0.09	0.025
Current pregnancy status <sup>3</sup>	−11.99 (−15.60, −8.39)	1.84	−0.27	<0.0001
RBP, <sup>4</sup> $\mu\text{mol/L}$	3.09 (1.77, 4.40)	0.67	0.19	<0.0001
Log ferritin, <sup>4</sup> $\mu\text{g/L}$	2.19 (0.86, 3.51)	0.67	0.13	0.001
Vitamin B-12, pmol/L	0.01 (0.00, 0.01)	0.003	0.09	0.026
Parity, <sup>3</sup> n	−0.64 (−1.25, −0.03)	0.31	−0.08	0.039

<sup>1</sup> Variables were added to the model in a forward stepwise conditional approach. Variables that were not significant in the model ( $P > 0.05$ ) and therefore not included in the model were age, BMI, log soluble transferrin receptor, log folate, log CRP, log AGP, and 2 other genetic hemoglobin disorders:  $\alpha^{3,7}$ -thalassemia trait and co-inherited hemoglobin E trait and  $\alpha^{3,7}$ -thalassemia trait. The correlation matrix and variance inflation factors showed no signs of multicollinearity between variables included in the model.  $R^2 = 0.32$ . Adjusted  $R^2 = 0.31$ . AGP,  $\alpha 1$  acid glycoprotein; B, unstandardized  $\beta$  coefficient; CRP, C-reactive protein; CS, Constant Spring; RBP, retinol binding protein.

<sup>2</sup> The largest change in  $R^2$  occurred with the addition of hemoglobin E homozygous group (13.4% change) and pregnancy status (9.7%) variables.

<sup>3</sup> Current pregnancy status and parity were self-reported by women.

<sup>4</sup> RBP and ferritin were adjusted for levels of subclinical inflammation by using AGP and CRP biomarkers before inclusion in model (28, 54). This was because log AGP and log CRP were not significant in the model when including the unadjusted values for RBP and ferritin.

(~1% based on ferritin). Comparatively, ferritin concentrations in the Cambodian women in our study were more than double those seen in a representative sample of Canadian women of similar age (ferritin of ~41  $\mu\text{g/L}$ ) (59) who presumably are consuming diets with a higher iron content. We expected that ferritin concentrations would be low in Cambodian women. This is because of iron-poor diets that consist mainly of rice (low in iron content) and lack of adequate quantities of iron-rich animal food sources. On the basis of self-reported data obtained from the women via questionnaires at the time of blood collection, women were not taking iron or micronutrient supplements, using iron cooking pots, or consuming iron-fortified fish or soy sauce.

Twenty-four-hour dietary recalls were collected and are currently being analyzed; however, preliminary data suggest that women were consuming little dietary iron. We speculate that naturally existing iron in ground water could be contributing to dietary intakes among women in Cambodia, as observed in other parts of Asia (60, 61).

The prevalence of other micronutrient deficiencies we assessed (folate and vitamins A, B-12 and B-6) ranged from very low to nonexistent. Unfortunately, we were not equipped to measure any biomarkers of riboflavin status in the women, which has been shown to be associated with anemia (9, 35). A recent unpublished study showed that riboflavin deficiency is prevalent (82%) among

**TABLE 4** Anemia prevalence and hematologic and other indicators in the 7 most commonly detected hemoglobin types in nonpregnant Cambodian women aged 18–45 y in rural Prey Veng province<sup>1</sup>

	AA	Hemoglobin E trait	$\alpha^{3,7}$ -Thalassemia trait	Hemoglobin E trait and $\alpha^{3,7}$ -thalassemia trait	Hemoglobin E homozygous group <sup>2</sup>	Hemoglobin CS trait	$\alpha^{3,7}$ -Thalassemia homozygous
Total, n (%)	195 (46.4)	56 (13.3)	50 (11.9)	35 (8.3)	31 (7.4)	15 (3.6)	7 (1.7)
Anemia, n (%)	22 (11.3)	21 (37.5)	15 (30.0)	11 (31.4)	30 (96.8)	3 (20.0)	4 (57.1)
Hemoglobin, g/L	130 $\pm$ 8.9	123 $\pm$ 8.6*	124 $\pm$ 9.3*	126 $\pm$ 8.9*	109 $\pm$ 7.3*	121 $\pm$ 7.0*	110 $\pm$ 13.4*
MCV, fL	87.3 $\pm$ 4.1	75.6 $\pm$ 4.1*	80.9 $\pm$ 5.3*	79.2 $\pm$ 3.3*	59.2 $\pm$ 3.8*	80.1 $\pm$ 5.3*	69.3 $\pm$ 4.0*
MCH, pg	28.3 $\pm$ 1.5	24.5 $\pm$ 1.4*	25.5 $\pm$ 1.8*	25.5 $\pm$ 1.2*	19.9 $\pm$ 1.4*	25.3 $\pm$ 1.9*	22.1 $\pm$ 1.1*
RDW, %	13.3 $\pm$ 1.1	14.6 $\pm$ 1.2*	13.8 $\pm$ 1.1*	14.3 $\pm$ 1.0*	17.6 $\pm$ 2.0*	13.7 $\pm$ 1.1*	15.4 $\pm$ 1.3*
RBC, $\times 10^{-9}/\text{L}$	4.6 $\pm$ 0.3	5.0 $\pm$ 0.4 *	4.9 $\pm$ 0.4*	4.9 $\pm$ 0.4*	5.4 $\pm$ 0.4*	4.8 $\pm$ 0.3	5.0 $\pm$ 0.7*
Ferritin, <sup>3</sup> $\mu\text{g/L}$	95.8 $\pm$ 56.2	85.1 $\pm$ 41.7	79.1 $\pm$ 44.0	92.7 $\pm$ 56.3	129 $\pm$ 90.6*	90.5 $\pm$ 37.0	101 $\pm$ 47.9
sTfR, mg/L	6.4 $\pm$ 1.9	7.0 $\pm$ 2.3*	6.8 $\pm$ 2.0	6.2 $\pm$ 1.5	9.5 $\pm$ 3.5*	9.5 $\pm$ 3.1*	6.5 $\pm$ 1.5
RBP, <sup>3</sup> $\mu\text{mol/L}$	2.54 $\pm$ 0.72	2.57 $\pm$ 0.57	2.49 $\pm$ 0.74	2.57 $\pm$ 0.82	2.56 $\pm$ 0.67	2.56 $\pm$ 0.69	2.47 $\pm$ 0.55
AGP, g/L	0.88 $\pm$ 0.27	0.88 $\pm$ 0.31	0.91 $\pm$ 0.28	0.83 $\pm$ 0.20	0.75 $\pm$ 0.18	0.90 $\pm$ 0.28	0.79 $\pm$ 0.24
CRP, mg/L	2.13 $\pm$ 4.00	1.62 $\pm$ 3.12	1.92 $\pm$ 4.26	2.07 $\pm$ 3.05	1.32 $\pm$ 1.43	1.45 $\pm$ 1.61	2.52 $\pm$ 5.81

<sup>1</sup> Values are frequencies (%) or means  $\pm$  SDs. Data are not shown for rare hemoglobin disorders affecting <7 women in each category. \*Pairwise comparisons show a significant difference ( $P < 0.05$ , least significant difference adjusted for multiple comparisons) between women with AA and women with a genetic hemoglobin disorder. AA, normal hemoglobin; AGP,  $\alpha 1$  acid glycoprotein; CRP, C-reactive protein; CS, Constant Spring; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; RBP, retinol binding protein; RDW, red blood cell distribution width; sTfR, soluble transferrin receptor.

<sup>2</sup> Hemoglobin E homozygous group indicates that an affected individual has a gene deletion on both  $\beta$ -genes (rather than a trait which indicates a gene deletion on only 1  $\beta$ -gene); this group in the table includes all homozygous forms of hemoglobin E including those co-inherited with other compound and heterozygous traits (hemoglobin CS trait,  $\alpha^{SEA}$ -thalassemia trait, etc).

<sup>3</sup> Values were corrected for subclinical inflammation by using AGP and CRP biomarkers (28, 54).

**TABLE 5** Prevalence of iron deficiency with and without anemia among 420 nonpregnant Cambodian women aged 18–45 y with and without genetic hemoglobin disorders<sup>1</sup>

	No genetic hemoglobin disorder, <i>n</i> (%)	Any genetic hemoglobin disorder, <i>n</i> (%)
Total sample	195 (46.4)	225 (53.6)
Iron deficiency (ferritin <15 µg/L or sTfR >8.3 mg/L)	22 (11.3)	60 (26.7)
Ferritin, depleted iron stores, <15 µg/L	5 (2.6)	4 (1.8)
sTfR, tissue iron deficiency, >8.3 mg/L	20 (10.3)	59 (26.2)
Anemia (hemoglobin <120 g/L)	22 (11.3)	102 (45.3)
Iron deficiency anemia (hemoglobin <120 g/L and ferritin <15 µg/L or sTfR >8.3 mg/L)	3 (1.5)	32 (14.2)
Ferritin <15 µg/L and hemoglobin <120 g/L	2 (1.0)	2 (<1.0)
sTfR >8.3 mg/L and hemoglobin <120 g/L	2 (1.0)	32 (14.2)

<sup>1</sup> sTfR, soluble transferrin receptor.

rural women in Prey Veng (*n* = 156) (KC Whitfield, unpublished data, 2014). Hence, this is an area that warrants further investigation.

Investigation of the biomarkers showed, most notably, that hemoglobin was significantly lower across all of the common genetic hemoglobin disorders. Ferritin and sTfR concentrations were elevated in individuals with certain genetic hemoglobin disorders. In neighboring Thailand, Thurlow et al. (45) also demonstrated that ferritin concentrations were significantly elevated in children (*n* = 567) with hemoglobin E. In our study, ferritin was significantly elevated among the hemoglobin E homozygous group (compared with women with normal hemoglobin) (*P* < 0.05). Similar to our findings, George et al. (44) found that sTfR concentrations were significantly elevated in Cambodian children (*n* = 2329) with hemoglobin E homozygous, hemoglobin E trait,  $\alpha$ -thalassemia trait, and the coinherited form of the latter 2. Evidence of elevated sTfR in pregnant Thai women (*n* = 113) with  $\alpha$ -thalassemia and hemoglobin E trait was also demonstrated (46). Elevated sTfR in individuals with hemoglobin disorders is thought to be a consequence of increased erythropoiesis stimulated by ineffective erythropoiesis (62). Furthermore, we found conflicting evidence of iron deficiency with elevated ferritin (high iron stores) and elevated sTfR concentrations (tissue iron deficiency) in women. We conclude that serum ferritin and sTfR are poor diagnostic indicators of iron deficiency in populations with hemoglobin disorders and inflammation. It highlights the importance of finding other methods to increase the validity of these biomarkers (potential use of correction factors) or alternatively exploring other biomarkers to assess iron deficiency in populations with a high prevalence of hemoglobin disorders and inflammation (potentially reticulocyte count).

The main limitations of the study were typical of cross-sectional surveys in which causation cannot be inferred and findings cannot be extrapolated beyond the geographical area and population group included in the model. We did not measure disease and infection, which could be contributing to anemia. Hookworm and malaria exist in Cambodia and are known to increase inflammation and contribute to anemia. However, despite the prevalence of chronic inflammation among women in our study (~25%), AGP (a biomarker of chronic inflammation) was not significant as a predictor of hemoglobin and therefore not included in our regression model.

We conclude that genetic hemoglobin disorders, rather than iron deficiency, are a major predictor of hemoglobin concentration in women aged 18–45 y in Prey Veng, Cambodia. Genetic hemoglobin disorders were prevalent in more than half of the women in our study and have serious potential consequences of morbidity. More work is necessary to collect nationwide data on

the frequencies hemoglobin disorders and to develop cheaper and simpler methods of screening and management. Future research is also warranted to investigate the causes of high serum ferritin concentrations and the potential risk of iron overload, particularly in women with hemoglobin E homozygous disorders. If iron deficiency is not a problem, then interventions such as iron fortification and supplementation should be reassessed and carefully monitored.

### Acknowledgments

We thank the following individuals for their contributions: Mr. Keith Porter, Helen Keller International, Cambodia country office; Mr. Chanthan Am, The National Institute of Public Health Laboratory, Cambodia; Dr. Jürgen Erhardt, ETH Zurich, Switzerland; Rika Aleliunas, The Child and Family Research Institute, Canada; and Mikaela K Barker, Aviva Rappaport, and Yazheng Amy Liu, The University of British Columbia, Canada. CDK and TJG designed the research, conducted the statistical analysis of the data, and had primary responsibility for the final content; CDK drafted the research protocol, conducted the research and managed the data, and drafted the research manuscript; TJG contributed to the review and editing of the protocol to the final stage and provided oversight and input into all aspects of the study; CDK, KCW, SIB, YL, AMD, SMV, HK, AT, JM, and TJG contributed to the data interpretation and to the review and editing of the manuscript to its final stage. All authors read and approved the final manuscript.

### References

1. World Health Organization; Centers for Disease Control and Prevention. Assessing the iron status of populations. 2nd ed. Geneva: WHO; 2007.
2. De Benoist B, McLean E, Egli I, Cogswell ME, editors. Worldwide prevalence of anaemia 1993–2005: WHO global database on anaemia. Geneva: WHO; 2008.
3. Ministry of Health, Government of Cambodia and Measure Demographic and Health Survey (DHS). Cambodia Demographic and Health Survey 2010. Calverton (MD): ICF Macro; 2011.
4. Allen LH. Anemia and iron deficiency: effects on pregnancy outcome. *Am J Clin Nutr* 2000;71(Suppl):1280S–4S.
5. Xiong X, Buekens P, Alexander S, Demainczuk N, Wollast E. Anemia during pregnancy and birth outcome: a meta-analysis. *Am J Perinatol* 2000;17:137–46.
6. Stoltzfus RJ, Mullany L, Black RE. Iron deficiency anaemia. In: Ezzati M, Lopez AD, Rodgers A, Murray CJL, editors. Comparative quantification of health risks: global and regional burden of disease attributable to selected major risk factors. Geneva: WHO; 2004. p. 163–209.

7. Haas JD, Brownlie T 4th. Iron deficiency and reduced work capacity: a critical review of the research to determine a causal relationship. *J Nutr* 2001;131(Suppl):676S–88S.
8. Fishman SM, Christian P, West KP. The role of vitamins in the prevention and control of anaemia. *Public Health Nutr* 2000;3:125–50.
9. Ma AG, Schouten EG, Zhang FZ, Kok FJ, Yang F, Jiang DC, Sun YY, Han XX. Retinol and riboflavin supplementation decreases the prevalence of anemia in Chinese pregnant women taking iron and folic acid supplements. *J Nutr* 2008;138:1946–50.
10. Zimmermann MB, Hurrell RF. Nutritional iron deficiency. *Lancet* 2007;370:511–20.
11. Bain BJ. Haemoglobinopathy diagnosis. 2nd ed. Oxford (UK): Blackwell Publishing; 2006.
12. Greer JP, Foerster J, Rodgers GM, Paraskevas F. Wintrobe's clinical hematology. 12th ed. Baltimore (MD): Lippincott Williams & Wilkins; 2009.
13. World Health Organization. The prevalence of anaemia in women: a tabulation of available information. 2nd ed. Geneva: WHO; 1992.
14. Tomkins A. Assessing micronutrient status in the presence of inflammation. *J Nutr* 2003;133(Suppl):1649S–55S.
15. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med* 2005;352:1011–23.
16. Gangat N, Wolanskyj AP. Anemia of chronic disease. *Semin Hematol* 2013;50:232–8.
17. Cambodia Council for Agricultural and Rural Development. Strategic framework for food security and nutrition in Cambodia 2008–2012. Phnom Penh (Cambodia): Government of Cambodia; 2011.
18. Gibson R. Principles of nutrition assessment. 2nd ed. New York: Oxford Press; 2005.
19. Crompton DWT, Nesheim MC. Nutritional impact of intestinal helminthiasis during the human life cycle. *Annu Rev Nutr* 2002;22:35–59.
20. Kasper MR, Blair PJ, Touch S, Sokhal B, Yasuda CY, Williams M, Richards AL, Burgess TH, Wierzbza TF, Putnam SD. Infectious etiologies of acute febrile illness among patients seeking health care in south-central Cambodia. *Am J Trop Med Hyg* 2012;86:246–53.
21. Vlieghe ER, Phe T, Smet BD, Veng HC, Kham C, Lim K, Koole O, Lynen L, Peetermans WE, Jacobs JA. Bloodstream infection among adults in Phnom Penh, Cambodia: key pathogens and resistance patterns. *PLoS ONE* 2013;8:e59775.
22. Muth S, Sayasone S, Odermatt-Biays B, Phompida S, Duong S, Odermatt P. *Schistosoma mekongi* in Cambodia and Lao People's Democratic Republic. *Adv Parasitol* 2010;72:179–203.
23. Hallberg L, Hulten L. Iron requirement, iron balance and iron deficiency in menstruating and pregnant women. In: Hallberg L, App NG, editors. *Iron nutrition in health and disease*. London: George Libbey; 1996.
24. Bothwell TH. Iron requirements in pregnancy and strategies to meet them. *Am J Clin Nutr* 2000;72(Suppl):257S–64S.
25. Harvey LJ, Armah CN, Dainty JR, Foxall RJ, John Lewis D, Langford NJ, Fairweather-Tait SJ. Impact of menstrual blood loss and diet on iron deficiency among women in the UK. *Br J Nutr* 2005;94:557–64.
26. Northrop-Clewes CA. Interpreting indicators of iron status during an acute phase response: lessons from malaria and human immunodeficiency virus. *Ann Clin Biochem* 2008;45:18–32.
27. Thurnham DI, McCabe GP. Influence of infection and inflammation on biomarkers of nutritional status with an emphasis on vitamin A and iron. In: World Health Organization report: priorities in the assessment of vitamin A and iron status in populations. Geneva: WHO; 2012.
28. Thurnham DI, McCabe LD, Haldar S, Wieringa FT, Northrop-Clewes CA, McCabe GP. Adjusting plasma ferritin concentrations to remove the effects of subclinical inflammation in the assessment of iron deficiency: a meta-analysis. *Am J Clin Nutr* 2010;92:546–55.
29. Beguin Y. Soluble transferrin receptor for the evaluation of erythropoiesis and iron status. *Clin Chim Acta* 2003;329:9–22.
30. Skikne BS. Serum transferrin receptor. *Am J Hematol* 2008;83:872–5.
31. Infusino I, Braga F, Dolci A, Panteghini M. Soluble transferrin receptor (sTfR) and sTfR/log ferritin index for the diagnosis of iron deficiency anemia: a meta analysis. *Am J Clin Pathol* 2012;138:642–9.
32. Kraemer K, Zimmerman MB, editors. Nutritional anemia. Basel (Switzerland): Sigh and Life Press; 2007.
33. de Benoist B. Conclusions of a WHO technical consultation on folate and vitamin B12 deficiencies. *Food Nutr Bull* 2008;29:S238–44.
34. Suharno D, West CE, Muhilal, Karyadi D, Hautvast JG. Supplementation with vitamin A and iron for nutritional anaemia in pregnant women in West Java, Indonesia. *Lancet* 1993;342:1325–8.
35. Powers HJ, Hill MH, Mushtaq S, Dainty JR, Majsak-Newman G, Williams EA. Correcting a marginal riboflavin deficiency improves hematologic status in young women in the United Kingdom (RIBOFEM). *Am J Clin Nutr* 2011;93:1274–84.
36. World Health Organization. Serum ferritin concentrations for the assessment of iron status and iron deficiency in populations. Geneva: WHO; 2011.
37. Ajayi OA, Nnaji UR. Effect of ascorbic acid supplementation on haematological response and ascorbic acid status of young female adults. *Ann Nutr Metab* 1990;34:32–6.
38. Mao X, Yao G. Effect of vitamin C supplementations on iron deficiency anemia in Chinese children. *Biomed Environ Sci* 1992;5:125–9.
39. Reinken L, Kurz R. Activity studies of an iron-vitamin B6 preparation for enteral treatment of iron deficiency anemia. *Int J Vitam Nutr Res* 1975;45:411–8.
40. Hisano M, Suzuki R, Sago H, Murashima A, Yamaguchi K. Vitamin B6 deficiency and anemia in pregnancy. *Eur J Clin Nutr* 2010;64:221–3.
41. Carnley BP, Prior JF, Gilbert A, Lim E, Devenish R, Sing H, Sarin E, Guhadasan R, Sullivan SG, Wise CA, et al. The prevalence and molecular basis of hemoglobinopathies in Cambodia. *Hemoglobin* 2006;30:463–70.
42. Fucharoen S, Weatherall DJ. Hemoglobin E disorders. In: Steinberg MH, Forget BG, Higgs DR, Weatherall DJ, editors. *Disorders of hemoglobin*. Cambridge (UK): Cambridge University Press; 2009.
43. Fucharoen S, Weatherall DJ. The hemoglobin E thalassemias. *Cold Spring Harb Perspect Med* 2012;2:a011734.
44. George J, Yiannakis M, Main B, Devenish R, Anderson C, An US, Williams SM, Gibson RS. Genetic hemoglobin disorders, infection, and deficiencies of iron and vitamin A determine anemia in young Cambodian children. *J Nutr* 2012;142:781–7.
45. Thurlow RA, Winichagoon P, Green T, Wasantwisut E, Pongcharoen T, Bailey KB, Gibson RS. Only a small proportion of anemia in northeast Thai schoolchildren is associated with iron deficiency. *Am J Clin Nutr* 2005;82:380–7.
46. Uaprasert N, Rojnuckarin P, Bhokaisawan N, Settapiboon R, Wacharaprechanont T, Amornsiriwat S, Sutcharitchan P. Elevated serum transferrin receptor levels in common types of thalassemia heterozygotes in Southeast Asia: a correlation with genotypes and red cell indices. *Clin Chim Acta* 2009;403:110–3.
47. Cogill B. Anthropometric indicators measurement guide. Revised ed. Washington: Food and Nutrition Technical Assistance (FANTA) Project; 2003.
48. Erhardt JG, Estes JE, Pfeiffer CM, Biesalski HK, Craft NE. Combined measurement of ferritin, soluble transferrin receptor, retinol binding protein and C-reactive protein by an inexpensive, sensitive, and simple sandwich enzyme-linked immunosorbent assay technique. *J Nutr* 2004;134:3127–32.
49. O'Brian S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. *J Clin Pathol* 1992;45:344–7.
50. Ubbink JB, Serfontein WJ, de Villiers LS. Stability of pyridoxal-5-phosphate semicarbazone: applications in plasma vitamin B6 analysis and population surveys of vitamin B6 nutritional status. *J Chromatogr* 1985;342:277–84.
51. Hartwell SK, Srisawang B, Kongtawelert P, Christian GD, Grudpan K. Review on screening and analysis techniques for hemoglobin variants and thalassemia. *Talanta* 2005;65:1149–61.
52. Giordano PC. Carrier diagnostics and prevention of hemoglobinopathies using capillary electrophoresis. 1st ed. Evry (France): Sebia; 2007.
53. Tan AS, Quah TC, Low PS, Chong SS. A rapid and reliable 7-deletion multiplex polymerase chain reaction assay for alpha-thalassemia. *Blood* 2001;98:250–1.
54. Thurnham DI, McCabe GP, Northrop-Clewes CA, Nestel P. Effects of subclinical infection on plasma retinol concentrations and assessment of prevalence of vitamin A deficiency: meta analysis. *Lancet* 2003;362:2052–8.



55. Chen J, Shao J. Nearest neighbor imputation for survey data. *J Off Stat* 2000;16:113–31.
56. Chang A. Online sample size calculator for multiple regression models [Internet]. Brisbane (Australia): Statstodo Trading Pty. Ltd.; c2013 [updated 2013 Mar; cited 2014 Jul 11]. Available from: [https://www.statstodo.com/SSizMReg\\_Pgm.php/](https://www.statstodo.com/SSizMReg_Pgm.php/).
57. Jack SJ, Ou K, Chea M, Chhin L, Devenish R, Dunbar M, Eang C, Hou K, Ly S, Khin M, et al. Effect of micronutrient sprinkles on reducing anemia: a cluster-randomized effectiveness trial. *Arch Pediatr Adolesc Med* 2012;166:842–50.
58. King JC. The risk of maternal nutritional depletion and poor outcomes increases in early or closely spaced pregnancies. *J Nutr* 2003;133 (Suppl):1732S–6S.
59. Cooper M, Greene-Finestone L, Lowell H, Levesque J, Robinson S. Iron sufficiency of Canadians. *Health Rep* 2012;23:41–8.
60. Merrill RD, Shamim AA, Ali H, Labrique AB, Schulze K, Christian P, West KP. High prevalence of anemia with lack of iron deficiency among women in rural Bangladesh: a role for thalassemia and iron in groundwater. *Asia Pac J Clin Nutr* 2012;21:416–24.
61. Merrill RD, Shamim AA, Ali H, Jahan N, Labrique AB, Schulze K, Christian P, West KP. Iron status of women is associated with the iron concentration of potable groundwater in rural Bangladesh. *J Nutr* 2011;141:944–9.
62. Rees DC, Williams TN, Maitland K, Clegg JB, Weatherall DJ. Alpha thalassemia is associated with increased soluble transferrin receptor levels. *Br J Haematol* 1998;103:365–9.