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The Homozygous Hemoglobin EE Variant Is Associated with Poorer Riboflavin Status in Cambodian Women of Reproductive Age

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ABSTRACT

Background: Riboflavin is required for erythropoiesis, which is increased in people with hemoglobinopathies due to increased hemolysis and erythrocyte turnover. Dietary intake and status of riboflavin is poor in Cambodia, where hemoglobinopathies are common.

Objective: We assessed the association between genetic hemoglobin disorders and riboflavin status in women of reproductive age in Cambodia.

Methods: Venous blood samples from 515 Cambodian women of reproductive age, 18–45 y, were analyzed for biomarker status of riboflavin [erythrocyte glutathione reductase activation coefficient (EGRac)], genetic hemoglobin (Hb) disorders, and hematological indices. Linear regression analysis was used to estimate the association between EGRac with Hb, ferritin, and Hb genotypes. EGRac was log transformed in the analyses, and the regression coefficients represent the geometric mean differences.

Results: Genetic Hb disorders were present in 57% of the population, with the homozygous hemoglobin E variant (Hb EE) occurring in ~10% of women ($n = 53$). Deficient (EGRac ≥1.40) or marginal riboflavin status (EGRac ≥1.30 and <1.40) was observed in 92% ($n = 475$) of women. The variant Hb EE genotype was associated with 18% (95% CI: 9%, 28%) higher geometric mean EGRac values than the normal Hb AA genotype ($P < 0.001$).

Conclusions: Although riboflavin biomarker deficiency or marginal status is widely prevalent in Cambodian women, lower riboflavin status was observed more frequently in women with the Hb EE genotype than in women with normal Hb AA. The relation between genetic Hb disorders and riboflavin warrants further investigation. This trial was registered at clinicaltrials.gov as NCT01593423 and NCT02481375. *J Nutr* 2020;150:1943–1950.

Keywords: Cambodia, EGRac, hemoglobin, hemoglobinopathy, riboflavin, women

Introduction

Genetic hemoglobin (Hb) disorders are the most prevalent monogenic diseases worldwide, affecting ~7% of the global population (1), with 90% occurring in low-income countries (2). Genetic Hb disorders, characterized as autosomal recessive heterozygous or homozygous mutations and/or deletions in the α- or β-globin genes of Hb, are divided into 2 main groups: thalassemia syndromes and structural Hb variants (3, 4). Thalassemia results from reduced α- or β-globin chain synthesis (referred to as α- or β-thalassemia, respectively), whereas structural Hb variants result from amino acid substitutions in the globin chains (3).

Hemoglobin disorders have been widely reported throughout Southeast Asia, with α-thalassemia and hemoglobin E (Hb E) variants being most prevalent (5–8). In rural areas of Cambodia, genetic Hb disorders have been shown to affect the majority (>50%) of women of reproductive age (9). These disorders increase the risk of anemia, due to decreased or defective Hb production (4), and/or increased erythropoiesis due to a shorter red blood cell half-life (10). Further, these disorders have been associated with altered biomarkers of iron status, namely ferritin and soluble transferrin receptor (sTfR) concentrations (11). This alteration of iron status biomarkers is important, as the diagnostic accuracy of these biomarkers is reduced in individuals with genetic Hb disorders, potentially resulting in the misdiagnosis of iron deficiency.

Riboflavin deficiency is common in lower-income countries (12), with rates of deficiency [erythrocyte glutathione reductase

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activation coefficient (EGRac) ≥1.40] of ~80% previously reported in women of reproductive age in urban and rural Cambodia (13). Riboflavin is commonly found in dairy products, eggs, meats, and other expensive and/or unavailable animal-source foods, and the amount of riboflavin is very low in white polished rice, a common staple food in Southeast Asia (14, 15). The foods that are the major contributors to the diet of rural Cambodian women are white rice, cereal grains, vegetables and fruit, and fish or shellfish (16), of which vegetables and fish or shellfish are among the richest sources of riboflavin (17).

Riboflavin deficiency may impair iron mobilization and absorption, Hb production, and red blood cell synthesis, thereby contributing to the development of anemia (12, 17, 18). Riboflavin status is most often determined using the EGRac assay, a functional measure of riboflavin status (19). This assay reflects the intracellular saturation of the glutathione reductase enzyme by flavin adenine dinucleotide (FAD), an essential coenzyme determined largely by dietary riboflavin intake (20).

The association between riboflavin deficiency and Hb genotype is not well established. Increased rates of erythropoiesis, as seen with certain hemoglobinopathies, may lead to elevated micronutrient needs and increased risk of riboflavin deficiency, especially if dietary intake is marginal (21). The presence of these traits and/or Hb mutations may have a significant influence on EGRac. Given the high prevalence of riboflavin deficiency and hemoglobinopathies in Cambodia, we aimed to determine whether riboflavin status differed by Hb genotype.

Methods

Study population

Data from Cambodian women of reproductive age (18–45 y) from 2 studies, 1 study conducted in residents of the Prey Veng province and the other in residents of the Kampong Chhnang province, were included in the current study (**Figure 1**). **Table 1** summarizes the characteristics of the study participants (total $n = 515$) included from the 2 datasets. Ethical approval for both studies 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 study participants by ink-stamped thumbprint upon enrollment in the study.

The first trial (the Fish on Farms Enhanced Homestead Food Production Trial, registered at clinicaltrials.gov as NCT01593423) was conducted in 2015–2018 in Prey Veng province, Cambodia (11). The primary aim was to evaluate the impact of enhanced homestead food production, with or without fishponds, on Hb concentration in women and young children in Cambodia. A total of 450 women were enrolled in the trial; EGRac measurement was conducted in a random subset of 253 women (Figure 1). Hb, mean corpuscular volume (MCV), and red blood cell distribution width (RDW) measures were available for 246 participants. Retinol-binding protein (RBP), C-reactive protein (CRP), ferritin, soluble transferrin receptor (sTfR), and α-1 acid glycoprotein (AGP) concentrations were available for 240 participants. For $n = 7$ and $n = 13$ participants, respectively, biochemical indices were not available due to missing samples.

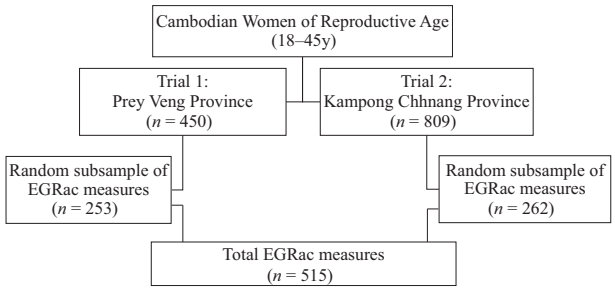

FIGURE 1 Flow diagram of Cambodian women of reproductive age through a secondary analysis of EGRac measures from 2 randomized control trials assessing the effect of nutrition interventions on Hb concentrations. EGRac, erythrocyte glutathione reductase activation coefficient; Hb, hemoglobin.

The second trial (the 2 × 2 Factorial Iron and Multiple Micronutrient Supplementation Trial, registered at clinicaltrials.gov as NCT02481375) was conducted in 2015 in Kampong Chhnang province, Cambodia (22). The primary aim was to evaluate the effect of 12 wk of iron supplementation with or without other micronutrients on Hb concentration in nonpregnant Cambodian women determined by screening as having anemia. A total of 809 women were enrolled in the trial; EGRac measurement was conducted on a random subset of 262 women (Figure 1). Complete hematological measurements (Hb, MCV, RDW, RBP, CRP, ferritin, sTfR, and AGP) and Hb genotype were available for all 262 women.

Blood collection, processing, and assessment

Venous blood samples (3-h fasting) were collected by trained phlebotomists in Cambodia. For the first trial, conducted in Prey Veng province, venous blood was collected in 3 evacuated tubes (Becton Dickinson); 2 tubes contained an anticoagulant (EDTA) and 1 tube did not. For the second trial, conducted in Kampong Chhnang, venous blood was collected in 2 evacuated tubes (Becton Dickinson), of which 1 tube contained an anticoagulant (EDTA). Samples were placed on ice and transported daily within 2–6 h of collection to the National Institute of Public Health Laboratory (NIPHL) in Phnom Penh for processing. A complete blood count was performed using an automated hematology analyzer (Sysmex, Sysmex Corp.) on fresh EDTA blood at NIPHL in Cambodia. Anemia was defined as Hb <120 g/L for nonpregnant women (23).

Blood samples in EDTA-free tubes were centrifuged at ~600 × *g* for 15 min at 4°C, and serum was collected and divided into aliquots. Serum was assessed for the following indicators by using a sandwich ELISA (24): ferritin (μg/L), sTfR (mg/L), AGP (g/L), CRP (mg/L), and RBP (μmol/L). Erythrocytes from an EDTA tube were washed 3 times with PBS and divided into aliquots for EGRac analyses. All samples were stored at −80°C until shipment to the appropriate laboratories for analysis.

EGRac was calculated as the ratio of FAD-stimulated to -unstimulated enzyme activity, indicating the degree of saturation with riboflavin (13). The activity of the enzyme glutathione reductase was measured in washed red cells before and after in vitro reactivation with FAD (13). Samples were batch analyzed in duplicate using a Daytona+ clinical chemistry analyzer (Randox Laboratories) at Ulster University in Northern Ireland. EGRac ratios are inversely related to riboflavin status: higher EGRac ratios are indicative of a lower saturation of samples with riboflavin's coenzyme FAD, and therefore poorer riboflavin status. Riboflavin status was defined as deficient for a finding of EGRac ≥1.40, marginal for EGRac ≥1.30 and <1.40, and sufficient for EGRac <1.30 (18, 25). Quality control (QC) was provided by repeated analysis of stored aliquots of pooled and characterized erythrocytes, with known EGRac values corresponding to adequate

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Abbreviations used: AGP, α-1 acid glycoprotein; CRP, C-reactive protein; EFSA, European Food Safety Authority; EGRac, erythrocyte glutathione reductase activation coefficient; FAD, flavin adenine dinucleotide; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; Hb E, hemoglobin E; Hb EE, homozygous hemoglobin E trait; MCV, mean corpuscular volume; NIPHL, National Institute of Public Health Laboratory; QC, quality control; RBP, retinol-binding protein; RDW, red blood cell distribution width; sTfR, soluble transferrin receptor.

TABLE 1 Characteristics of the study participants included in the datasets from 2 Cambodian provinces[1]

	Prey Veng province	Kampong Chhnang province
Total, *n* (%)	253 (49)	262 (51)
Age, y	31 ± 7	30 ± 8
EGRac	1.8 (1.6, 2.2)	1.8 (1.5, 2.1)
1.3 ≤ EGRac <1.4, *n* (%)	17 (7)	28 (11)
EGRac ≥1.4, *n* (%)	217 (86)	213 (81)
Hb, g/L	124.9 ± 11.1[2]	116.2 ± 12.8
Anemia, Hb <120 g/L, *n* (%)	75 (30)[2]	152 (58)
MCV, fL	81.0 (75.0, 86.1)[2]	77.0 (70.6, 83.2)
RDW, %	14.0 (13.0, 15.0)[2]	14.1 (13.1, 16.1)
Ferritin, μg/L	92.9 (60.2, 139.9)[3]	42.1 (18.8, 84.9)
sTfR, mg/L	6.3 (5.2, 7.8)[3]	5.7 (4.8, 7.6)
RBP, μmol/L	2.1 (1.7, 2.7)[3]	1.6 (1.3, 1.9)
AGP, g/L	0.7 (0.6, 0.9)[3]	0.6 (0.5, 0.7)
CRP, mg/L	0.8 (0.3, 1.7)[3]	0.4 (0.2, 0.9)
Genetic Hb disorders, *n* (%)		
Hb E trait (AE)	24/253 (9)	64/262 (24)
α-thalassemia trait	30/253 (11)	58/262 (22)
Hb E trait (AE) and α-thalassemia trait	23/253 (9)	43/262 (16)
Hb EE	18/253 (7)	15/262 (6)
Hb EE and α-thalassemia trait	3/253 (1)	17/262 (6)

[1]Values are frequencies (%), mean ± SD, or median (IQR). Total $n = 515$ women. AGP, α-1 acid glycoprotein; CRP, C-reactive protein; EGRac, erythrocyte glutathione reductase activation coefficient; Hb, hemoglobin; Hb EE, homozygous hemoglobin E; MCV, mean corpuscular volume; RBP, retinol binding protein; RDW, red blood cell distribution width; sTfR, soluble transferrin receptor.
[2]$n = 246$.
[3]$n = 240$.

and deficient status. For EGRac analysis, the interassay CVs were 2.7% and 4.1% for the adequate and deficient QCs, respectively.

Genetic Hb disorders were identified using methods of Hb electrophoresis and PCR (26). Capillary Hb electrophoresis was conducted using a Sebia MINICAP analyzer (Hb E program) by a trained external consultant at NIPHL in Cambodia. This automated technique quantifies the different types of Hb in blood for interpretive diagnosis and can detect normal Hb (Hb A and Hb A_2) and Hb variants (Hb E). At the Molecular Genetics Laboratory at BC Children's Hospital in Canada, genomic DNA was extracted from the buffy coat by using a QiaAmp blood DNA kit, and a multiplex PCR assay (27) was used to detect heterozygosity, homozygosity, and the presence of α-thalassemia.

Statistical analyses

Descriptive statistics were computed and are presented as mean ± SD, median (IQR), or *n* (%). Chi-square analysis was applied to determine statistically significant associations between Hb genotypes and riboflavin status (deficient: EGRac ≥1.4; marginal: EGRac ≥1.30 and <1.40; sufficient: EGRac <1.30), and anemia (Hb <120 g/L). Kruskal–Wallis H tests were applied to compare nonnormally distributed median hematological values (EGRac, Hb, MCV, RDW, RBP, CRP, ferritin, sTfR, and AGP) by Hb genotype. If statistically significant differences were detected among medians, the Dunn's multiple comparison test was applied with a Bonferroni correction. Mann–Whitney U tests were applied to compare nonnormally distributed median hematological values (Hb, MCV, RDW, RBP, CRP, ferritin, sTfR, and AGP) by riboflavin status (sufficient: EGRac <1.30, or marginal/deficient: EGRac ≥ 1.30) among women with wildtype Hb AA and homozygous Hb EE genotypes.

Generalized linear regression was used to assess associations of several of independent variables, with EGRac as a continuous outcome variable. The distribution of EGRac was right skewed; therefore, both the independent and outcome variables were natural log transformed before inclusion in the regression models. In a model with a log-transformed outcome variable, the β coefficients are interpreted in terms of the proportional change in the outcome variable. To calculate the proportional change, we exponentiated ($\exp^{\beta 1}$) the unstandardized β coefficients to estimate the expected proportional change in the geometric mean of the outcome variable (28). The proportional change in the geometric mean of the independent variable in the model was then divided by the geometric mean of the reference to provide a geometric mean ratio (28). The geometric mean ratios and their 95% CIs are reported. A ratio >1 indicates a positive association between the explanatory variable and outcome variable, and a ratio <1 indicates an inverse association (29). Ratios are interpreted somewhat similarly to RRs. For example, a 1-unit increase in the explanatory variable is associated with a 10% higher geometric mean value of the outcome variable.

Hematological variables (MCV, RDW, RBP, CRP, ferritin, sTfR, and AGP) were selected for inclusion in the model based on a crude compared with an adjusted change-in-estimate of ≥10% (30, 31).

A 2-sided significance level of 0.05 was applied. Data were analyzed with Stata IC/16.0 for Mac (Stata Corp).

Results

In total, 515 Cambodian women, aged 30 ± 8 y, were included in this study. Genetic Hb genotypes ($n = 515$), complete blood counts ($n = 508$), and extended hematological measures (RBP, CRP, ferritin, sTfR, and AGP; $n = 502$) are presented (Table 1). Genetic Hb disorders were present in 57% ($n = 295$) of the population (Table 1). Hb E trait and α-thalassemia trait were the most common genotypes, each occurring in 17% ($n = 88$) of the population (Table 1). The Hb E trait in combination with α-thalassemia trait occurred in 66 women (13%), and the homozygous hemoglobin E variant (Hb EE), with or without the α-thalassemia trait, occurred in 53 women (10%) (Table 1).

Of all of the women in the present study, 45% had anemia (Hb <120 g/L) with rates of anemia as high as 83% in individuals with Hb EE (**Table 2**). Hb concentrations were

TABLE 2 Anemia prevalence and hematological indicators in the 5 most commonly detected Hb types among 515 Cambodian women[1]

	Normal Hb (AA)	α-Thalassemia trait	Hb E trait (AE)	Hb E trait (AE) & α-thalassemia trait	Hb EE[2]
Total, *n* (%)	220 (43)	88 (17)	88 (17)	66 (13)	53 (10)
EGRac	1.7 (1.5, 2.1)[a]	1.7 (1.5, 2.1)[a]	1.9 (1.5, 2.3)[a]	1.8 (1.6, 2.2)[a]	2.0 (1.7, 2.8)[b]
1.3 ≤ EGRac <1.4, *n* (%)	20 (9)	12 (14)	6 (7)	4 (6)	3 (6)
EGRac ≥ 1.4, *n* (%)	183 (83)	71 (81)	70 (80)	58 (88)	48 (91)
Hb, g/L	126 (119, 133)[a]	119 (112, 127)[b]	119 (111, 124)[b]	121 (112, 126)[b]	111 (107, 117)[c]
Anemia, Hb <120 g/L, *n* (%)[3]	61/216 (28)	44/87 (51)	48/87 (55)	30/65 (46)	44/53 (83)
MCV, fL	85.3 (81.0, 88.7)[a]	79.0 (71.0, 82.9)[b]	75.8 (73.3, 78.0)[b]	77.3 (74.6, 81.7)[b]	60.0 (58.0, 62.0)[c]
RDW, %	13.0 (12.7, 14.0)[a]	14.0 (13.0, 15.3)[b]	14.0 (13.6, 15.0)[b]	14.0 (13.1, 15.2)[b]	17.2 (16.0, 18.5)[c]
Ferritin, μg/L	77.6 (33.7, 118.9)	66.0 (36.0, 114.1)	62.5 (33.8, 99.8)	51.0 (20.9, 104.6)	84.2 (39.0, 144.7)
sTfR, mg/L	6.0 (5.1, 7.8)[a]	6.0 (4.8, 7.7)[a]	5.4 (4.7, 6.7)[b]	5.3 (4.9, 7.3)[a]	7.5 (6.0, 11.0)[c]
RBP, μmol/L	1.9 (1.5, 2.4)	1.7 (1.3, 2.4)	1.7 (1.5, 2.1)	1.8 (1.4, 2.3)	1.8 (1.4, 2.2)
AGP, g/L	0.7 (0.6, 0.9)[a]	0.6 (0.5, 0.8)[a]	0.6 (0.5, 0.8)[b]	0.6 (0.5, 0.8)[b]	0.6 (0.5, 0.9)[a]
CRP, mg/L	0.6 (0.2, 1.4)	0.6 (0.2, 1.7)	0.5 (0.3, 1.4)	0.4 (0.2, 0.9)	0.5 (0.2, 1.6)

[1]Values are frequencies (%), means ± SDs, or medians (IQRs). AGP, α-1 acid glycoprotein; CRP, C-reactive protein; EGRac erythrocyte glutathione reductase activation coefficient; Hb, hemoglobin; Hb EE, homozygous hemoglobin E; MCV, mean corpuscular volume; RBP, retinol binding protein; RDW, red blood cell distribution width; sTfR, soluble transferrin receptor.
[2]Hb EE group indicates that an affected individual has a gene deletion on both β-genes (rather than a trait which indicates a gene deletion on only 1 β-gene); this group in the table includes all homozygous forms of Hb E, including those coinherited with or without α-thalassemia. Labeled values in a row without a common letter differ, $P < 0.05$.
[3]Chi-square analysis shows a significant difference between prevalence of anemia among Hb genotypes ($P < 0.05$).

inversely correlated with EGRac measures ($r = -0.19$, $P < 0.0001$). Pairwise comparisons, adjusted for multiple comparisons, revealed significant differences in Hb concentrations, MCVs, and RDWs (%) among all women with Hb disorder genotypes compared with those with normal (wildtype) Hb AA ($P < 0.05$) (Table 2). Hematological indices, however, did not differ by riboflavin status (either sufficient or marginal/deficient) in women with the wildtype Hb AA or Hb EE genotypes (Table 3).

Riboflavin deficiency (EGRac ≥1.40) and marginal riboflavin status (EGRac ≥1.30 and <1.40) were detected in 430 (83%) and 45 (9%) participants, respectively. Pairwise comparisons, adjusted for multiple comparisons, illustrated that women with the Hb EE genotype with or without α-thalassemia (10% of women) were the only genotype group who had significantly higher median (IQR) EGRac scores than women with the wildtype Hb AA genotype [2.0 (1.7, 2.8) compared with 1.7 (1.5, 2.1); $P < 0.05$] (Table 2; **Figure 2**). This result was further quantified in the generalized linear regression model with geometric mean ratios, in which women with the Hb EE genotype had an 18% (95% CI: 9%, 28%) higher EGRac than women with the Hb AA genotype (**Table 4**).

Discussion

To our knowledge, this is the first study to explore the association between riboflavin status and genetic Hb disorders. Although both hemoglobinopathies and riboflavin deficiency were prevalent in this cohort of Cambodian women of reproductive age (identified in 57% and 84% of women, respectively), only Hb EE genotype (with or without α-thalassemia; representing 10% of the cohort) was significantly associated with poorer riboflavin status. Women with the Hb EE genotype had an 18% higher EGRac, which is indicative of

TABLE 3 Hematological indicators by riboflavin status among Cambodian women with wildtype Hb (AA) and homozygous Hb EE genotypes[1]

	Normal Hb (AA) (*n* = 220)		Hb EE[2] (*n* = 53)	
	Riboflavin sufficient[2] (*n* = 17)	Riboflavin marginal or deficient[3] (*n* = 203)	Riboflavin sufficient[3] (*n* = 2)	Riboflavin marginal or deficient[4] (*n* = 51)
Hb, g/L	127 (117, 132)	126 (119, 133)	113 (102, 124)	111 (107, 117)
MCV, fL	87 (86, 90)	85 (80, 89)	63 (59, 67)	60 (58, 62)
RDW, %	13.0 (12.0, 13.0)	13.0 (12.7, 14.0)	16.5 (15.9, 17.0)	17.2 (16.0, 18.6)
Ferritin, μg/L	71.6 (54.5, 98.8)	79.0 (32.3, 119.2)	97.6 (44.8, 150.3)	84.2 (33.9, 144.7)
sTfR, mg/L	5.9 (5.4, 7.9)	6.1 (5.1, 7.8)	13.8 (5.2, 22.4)	7.5 (6.1, 10.8)
RBP, μmol/L	1.8 (1.5, 2.3)	1.9 (1.5, 2.4)	1.6 (1.5, 1.7)	1.8 (1.4, 2.3)
AGP, g/L	0.7 (0.5, 0.7)	0.7 (0.6, 0.9)	0.4 (0.2, 0.5)	0.7 (0.5, 0.9)
CRP, mg/L	0.4 (0.1, 0.8)	0.6 (0.3, 1.5)	0.2 (0.2, 0.2)	0.5 (0.2, 1.7)

[1]Values are medians (IQRs). AGP, α-1 acid glycoprotein; CRP, C-reactive protein; Hb, hemoglobin; MCV, mean corpuscular volume; RBP, retinol binding protein; RDW, red blood cell distribution width; sTfR, soluble transferrin receptor.
[2]Hb E homozygous group indicates that an affected individual has a gene deletion on both β-genes (rather than a trait which indicates a gene deletion on only one β-gene); this group in the table includes all homozygous forms of Hb E including those co-inherited with or without α-thalassemia).
[3]Women with EGRac <1.30.
[4]Women with EGRac ≥1.30.

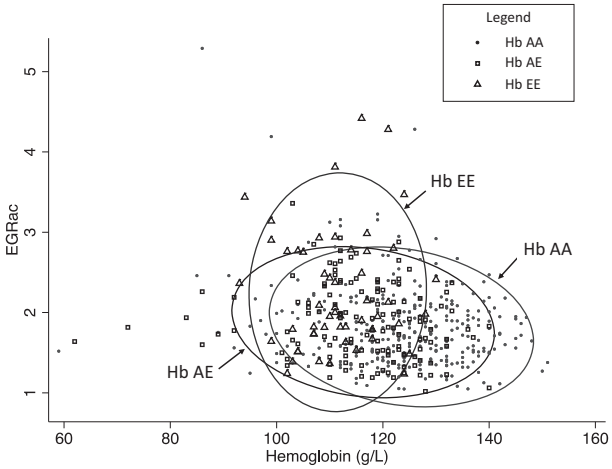

FIGURE 2 Relation between EGRac and hemoglobin concentration, by hemoglobin A and E genotypes, among 515 Cambodian women. Ellipses represent mean-centered 95% CIs for each genotype. EGRac, erythrocyte glutathione reductase activation coefficient; Hb, hemoglobin; Hb AA, normal hemoglobin; Hb AE, heterozygous hemoglobin E; Hb EE, homozygous hemoglobin E.

poorer riboflavin status, than women with the normal Hb AA (wildtype) genotype.

The interplay between riboflavin deficiency and Hb E disorders is complex and likely cyclical in nature. Individuals with the Hb EE genotype are expected to have higher erythrocyte turnover, due to the increased erythropoiesis and shorter cell lifespans that can accompany hereditary hemolytic anemia (32). Thus, higher requirements for nutrients that are necessary for erythropoiesis, including riboflavin and other nutrients, including vitamin A, zinc, selenium, folate, vitamin B-6 and vitamin B-12, which work in tandem for normal production of red blood cells, are likely required (12, 33, 34). Furthermore, deficiency of riboflavin can contribute to significant alterations in erythropoiesis (35). Studies performed as early as the 1950s have demonstrated that riboflavin deficiency–associated anemia in humans is characterized by reticulocytopenia, significant decreases in Hb concentrations, and increases in proerythroblasts (17, 36). Riboflavin deficiency has also been associated with alterations in iron utilization in humans. Among 1253 Chinese adults, iron intake was only related to the probability of anemia (adjusted for age, smoking, energy intake, dietary patterns, education, income, BMI, and hypertension) when riboflavin intake was <1.4 mg/d (P-trend = 0.016) (37). Animal studies have suggested that riboflavin deficiency can also decrease mobilization of iron from ferritin stores and increase rates of gastrointestinal iron loss (17). Thus, the effect of riboflavin deficiency on erythropoiesis is likely multifactorial in nature.

In the context of riboflavin status, the differences seen in this study among Hb genotypes may be further explained by the pathophysiology of the different genetic variants. Hb EE has been noted to present as mild hemolytic anemia and/or microcytosis, whereas heterozygous Hb E is often symptomless (38). These findings may provide a rationale for the significantly higher EGRac values in individuals with Hb EE than in individuals with homozygous Hb A. In individuals with α-thalassemia, the structure of Hb α-globin chains is maintained but α-globin chains are reduced in number or absent, leading to different hematological responses than those that occur in individuals with structural hemoglobinopathies, such as Hb E variants (32). This pathophysiology is associated more with ineffective erythropoiesis than with the occurrence of hemolysis (32). The presence of the α-thalassemia trait has a limited effect in combination with normal homozygous Hb A genotypes, as only a defect of the 3 α-globin gene has been associated with decreases in α-globin output (38). Further, when the α-thalassemia trait is present in combination with Hb E, it has been shown to decrease total Hb E concentrations due to increased competition between the β^A and β^E subunits for limited amounts of α subunits (39). This effect may limit the clinical manifestations of the Hb E trait, which may be the reason why the α-thalassemia trait, in combination with the Hb E trait, was not associated with increased EGRac in comparison with normal Hb.

Other researchers have hypothesized that altered riboflavin metabolism may also contribute to poorer riboflavin status in individuals with hemoglobinopathies such as β-thalassemia, a genotype noted to be associated with similar but more severe clinical features than Hb E (40). A previous study by Anderson et al. (41) in Italy suggested that in adults with heterozygous β-thalassemia (n = 72), the rates of riboflavin deficiency (EGRac >1.40) were significantly higher than those in relatives and spouses without β-thalassemia (n = 48; P < 0.001), despite a lack of difference in dietary intake. This

TABLE 4 Factors associated with EGRac among 515 Cambodian women[1]

	GMR[2]	95% CI	P value	Standardized GMR[2]	95% CI
Hb, g/L	0.997	0.995, 0.999	0.004	0.86	0.77, 0.95
Ferritin, μg/L	1.00	1.00, 1.00	0.21	1.06	0.97, 1.15
Hb genotypes (reference: normal Hb AA)					
α-Thalassemia trait	1.02	0.95, 1.09	0.57	1.07	0.82, 1.33
Hb E trait (AE)	1.01	0.95, 1.08	0.69	1.05	0.80, 1.31
Hb E trait (AE) & α-thalassemia trait	1.03	0.96, 1.11	0.38	1.12	0.84, 1.4
Hb EE[3]	1.18	1.09, 1.28	<0.001	1.63	1.31, 1.95
(Constant)	2.42	1.91, 3.05	NA	NA	NA

[1] Values are GMR (95% CI) unless otherwise indicated. Multivariable linear regression was used to assess associations of a number of independent explanatory variables with EGRac as the continuous outcome variable. Unstandardized β coefficients were calculated for outcome variables (the natural log of the EGRac), which were exponentiated ($\exp^{\beta 1}$), resulting in the GMR. EGRac, erythrocyte glutathione reductase activation coefficient; GMR, geometric mean ratio; Hb, hemoglobin; Hb EE, homozygous hemoglobin E; NA, not applicable.

[2] GMR were also calculated for the standardized β coefficients, to produce standardized GMRs, which present more easily interpretable ratios for which each independent explanatory variable has a variance of 1.

[3] Hb E homozygous group indicates that an affected individual has a gene deletion on both β-genes (rather than a trait which indicates a gene deletion on only one β-gene); this group in the table includes all homozygous forms of Hb E, including those coinherited with or without α-thalassemia.

finding was hypothesized to be due to an increased diversion of FAD to FAD-dependent methemoglobin reductases in those individuals with heterozygous β-thalassemia (41). Although this process has not been demonstrated in individuals with Hb E genotypes, it is possible that a similar mechanism contributes to higher EGRac in this population.

Research that further elucidates the relation between hemoglobinopathies and poor riboflavin status is relevant due to the high rates of riboflavin deficiency in Southeast Asia. Suboptimal riboflavin status often goes undiagnosed in low-income settings as EGRac and/or other biochemical assessments of riboflavin are not common or readily available. Further, the clinical symptoms of riboflavin deficiency (ariboflavinosis) can significantly overlap with those of other nutrient deficiencies, such as anemia in iron, vitamin A, vitamin B-12, and folate deficiencies; neurological degeneration in vitamin B-12 deficiency; and scaly dermatitis and inflamed eyes in biotin deficiency (42). While riboflavin deficiency itself is not fatal, it may have wide-ranging metabolic effects as riboflavin is a key coenzyme in the metabolism of folate, vitamin B-12, and vitamin B-6 (17). Further, emerging evidence has suggested that riboflavin may have other physiological effects, as supplementation has been shown to significantly reduce blood pressure in individuals with the methylenetetrahydrofolate reductase (MTHFR) 677TT genotype, which is estimated to affect 10% of the worldwide population and up to 30% of individuals in some populations (19, 25, 43). This finding highlights the importance of an integrative approach which incorporates riboflavin assessment in nutritional program planning and research endeavors in the region of Southeast Asia.

The use of EGRac measurements as the optimal means of assessing riboflavin status and the use of cutoffs to determine the adequacy of this nutrient have continued to be controversial. The European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies has suggested that urinary riboflavin excretion curves can be used as a biomarker of short-term riboflavin status, as EGRac has limitations to its use, such as in those with glucose-6-phosphate dehydrogenase (G6PD) deficiency (44). It has been noted, however, that urinary riboflavin excretion is not a sensitive biomarker of riboflavin intakes <1.1 mg/d (44). In Cambodia, it has been estimated that the daily per capita riboflavin intake is 0.56 mg/d based on the average ~2000-kcal/d diet (45), which illustrates that in this context, urinary riboflavin excretion may be limited in its usefulness as a biomarker. EGRac has been noted to be sensitive to riboflavin intake <1.0 mg/d, although it is not sensitive to variations in riboflavin intake (44). Furthermore, in rural Cambodia G6PD deficiency has been shown to effect only 5.8% of the female population (46), illustrating that this deficiency may have had only a limited impact on our study population.

We have applied cutoffs for EGRac in this study that have been widely used to determine riboflavin inadequacy, but there is a large degree of variation in applicable cutoffs. Previous studies have used EGRac cutoffs that range from 1.20 to 1.70 to determine low riboflavin or riboflavin deficiency (47–51). The EFSA panel considers that, on the basis of urinary excretion studies in adults, an EGRac of <1.30 can be applied to define riboflavin adequacy (44), which is in keeping with the cutoffs for marginal status and deficiency we have applied in this study. Further work to determine the most appropriate cutoffs for EGRac is warranted.

As a secondary analysis of data from 2 large randomized clinical trials, this study is limited by a lack of complete dietary riboflavin intake data. In the first trial (the Fish on Farms Enhanced Homestead Food Production Trial), conducted in Prey Veng province, endline dietary analysis was conducted among women of reproductive age ($n = 429$), and the results indicated that the mean (95% CI) intake of riboflavin, but not protein, was higher among women in the enhanced home food production program with aquaculture ($n = 143$) than among controls ($n = 140$; mean: 1.07; 95% CI: 0.96, 1.18 mg/d and 0.90; 0.80, 0.99 mg/d, respectively; $P < 0.05$) (52). Despite improvements in riboflavin intake in the intervention group, the mean riboflavin intakes reported in this population fell below the recommended dietary allowance for women between the ages of 19 and 70 y (1.1–1.3 mg/d) (15). The second trial (the 2 × 2 Factorial Iron and Multiple Micronutrient Supplementation Trial), however, which was conducted in 2015 in Kampong Chhnang, did not include dietary analysis, which limited the ability to integrate both biochemical and dietary indices in the analyses of these 2 study subgroups. This study was also limited by the cross-sectional design in which subsections of the 2 trial populations were randomly selected to have EGRac analyses completed.

Overall, homozygous Hb E has emerged as a factor associated with higher EGRac scores in Cambodian women of reproductive age. The potential relation between the Hb E genotype and poor riboflavin status has not been previously described. Further investigation of this potential relation, in other populations with high rates of both genetic Hb disorders and poor riboflavin status, is warranted.

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