The Journal of Nutrition
Community and International Nutrition

Genetic Hemoglobin Disorders, Infection, and Deficiencies of Iron and Vitamin A Determine Anemia in Young Cambodian Children[1–3]

Joby George,[4] Miriam Yiannakis,[5] Barbara Main,[6] Robyn Devenish,[7] Courtney Anderson,[6] Ung Sam An,[8] Sheila M. Williams,[9] and Rosalind S. Gibson[10*]

[4]World Vision Cambodia, Phnom Penh, Cambodia; [5]Nutrition Center of Expertise, World Vision International, Mississauga, Ontario; [6]World Vision Canada, Mississauga, Ontario, Canada Mississauga, Ontario, Canada; [7]Laboratory Medicine Unlimited, Phnom Penh, Cambodia; [8]National Institute of Public Health, Phnom Penh, Cambodia; and Departments of [9]Preventive and Social Medicine and [10]Human Nutrition, University of Otago, Dunedin, New Zealand

Abstract

In Cambodia, many factors may complicate the detection of iron deficiency. In a cross-sectional survey, we assessed the role of genetic hemoglobin (Hb) disorders, iron deficiency, vitamin A deficiency, infections, and other factors on Hb in young Cambodian children. Data on sociodemographic status, morbidity, and growth were collected from children (n = 3124) aged 6 to 59 mo selected from 3 rural provinces and Phnom Penh municipality. Blood samples were collected (n = 2695) for complete blood count, Hb type (by DNA analysis), ferritin, soluble transferrin receptor (sTfR), retinol-binding protein (RBP), C-reactive protein, and α_1-acid glycoprotein (AGP). Genetic Hb disorders, anemia, and vitamin A deficiency were more common in rural than in urban provinces ($P < 0.001$): 60.0 vs. 40.0%, 58.2 vs. 32.7%, and 7.4 vs. 3.1%, respectively. Major determinants of Hb were age group, Hb type, ferritin, sTfR, RBP, AGP >1.0 g/L ($P < 0.001$), and rural setting ($P < 0.05$). Age group, Hb type, RBP, elevated AGP, and rural setting also influenced ferritin and sTfR ($P < 0.02$). Multiple factors affected anemia status, including the following: age groups 6–11.99 mo (OR: 6.1; 95% CI: 4.3, 8.7) and 12–23.99 mo (OR: 2.7; 95% CI: 2.1, 3.6); Hb type, notably Hb EE (OR: 18.5; 95% CI: 8.5, 40.4); low ferritin (OR: 3.2; 95% CI: 2.2, 4.7); elevated AGP (OR: 1.4; 95% CI: 1.2,1.7); rural setting (OR: 2.3; 95% CI: 1.7, 3.1); low RBP (OR: 3.6; 95% CI: 2.2, 5.9); and elevated sTfR (OR: 2.1; 95% CI: 1.7, 2.7). In Cambodia, where a high prevalence of genetic Hb disorders exists, ferritin and sTfR are of limited use for assessing the prevalence of iron deficiency. New low-cost methods for detecting genetic Hb disorders are urgently required. J. Nutr. 142: 781–787, 2012.

Introduction

Anemia is a major and persistent public health problem in Cambodia, where the rate of anemia among children aged 6–59 mo is still >50% (1), with severe potential adverse health consequences. Nevertheless, there is limited information in Cambodia on the relative contributions of factors known to be associated with childhood anemia. Nutritional iron deficiency is often assumed to be the major etiologic factor (2), in part because microcytic, hypochromic anemia predominates. However, anemia of this type is also associated with both vitamin A deficiency (3) and genetic Hb[11] disorders that affect the structure, function and/or production of Hb (4). The occurrence of both of these conditions is well documented in Cambodian children (5–8).

Comprehensive community-based investigations of inherited Hb disorders in Cambodia are limited. Two genetically distinct variants are common: Hb E and α-thalassemia (6,9); their frequency varies with geographic region. Hb E disease arises from a genetic alteration in the physical structure of Hb, specifically a single amino acid substitution in one of the β-globin chains. In α-thalassemia, mutations involving deletion of one or more α-globin genes cause impaired globin chain synthesis. Combinations of α-thalassemia variants with Hb E also occur. The clinical features of genetic Hb disorders vary with the severity of the genetic defect and their effect on Hb structure and function. Some present as mild-to severe anemia, whereas others are associated with multiple clinical complications that may sometimes be severe enough to cause death in utero (10). Some also affect the biomarkers of iron status, limiting their usefulness for detecting iron deficiency (4,8).

Parasitic infections may also be implicated in the etiology of anemia in Cambodia. In the past, malaria was widespread in

[1]Supported by World Vision Canada.
[2]Author disclosures: J. George, M. Yiannakis, B. Main, R. Devenish, C. Anderson, U. S. An, S. M. Williams, and R. S. Gibson, no conflicts of interest.
[3]Supplemental Figure 1 and Supplemental Table 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[11] Abbreviations used: AGP, α_1-acid glycoprotein; CDHS, Cambodia Demographic and Health Survey; CRP, C-reactive protein; Hb, hemoglobin; IDA, iron-deficiency anemia; NIPHL, National Institute of Public Health Laboratory; RBP, retinol binding protein; RDW, red cell distribution width; sTfR, soluble transferrin receptor.
* To whom correspondence should be addressed. E-mail: rosalind.gibson@stonebow.otago.ac.nz or rosalind.gibson@otago.ac.nz.

Manuscript received July 18, 2011. Initial review completed August 31, 2011. Revision accepted February 9, 2012.
First published online February 29, 2012; doi:10.3945/jn.111.148189.

Cambodia but now exists in only a few provinces (11). In contrast, intestinal helminth infections such as hookworm and *Ascaris lumbricoides* continue to be common, with infection rates in children >50% in some rural settings (12). In addition to depleting body iron through blood loss, helminth infections may also exacerbate the risk of other micronutrient deficiencies by reducing digestion and absorption and by enhancing nutrient losses (e.g., vitamin A) (13).

Clearly, a variety of nutritional and nonnutritional factors including genetic Hb disorders, parasitic infections, and socioeconomic inequalities in Cambodia could influence the prevalence of anemia. However, their relative importance has not been examined. Furthermore, the extent to which genetic Hb variants complicate the identification of iron deficiency is uncertain. Therefore, the objectives of this cross-sectional survey in Cambodian preschool children aged 6 to 59 mo were as follows: *1*) to assess and compare the prevalence of anemia in rural and urban settings; *2*) to investigate the factors that might predispose them to anemia with particular emphasis on the role of genetic Hb disorders, iron and vitamin A deficiency, infections, and socioeconomic status; and *3*) to examine the extent to which the genetic Hb disorders confound the identification of iron deficiency.

Participants and Methods

Study design and participant selection. A community-based, cross-sectional survey was conducted in children from 3 rural provinces in Cambodia where World Vision Cambodia was working (Battambang, Kampong Thom, Preah Vihear) and in the Municipality of Phnom Penh (excluding the Central Operating District). Children were selected by using a 2-stage cluster sampling technique. In the first stage, 108 villages (Primary Sampling Units) were randomly selected from each province on the basis of probability proportional to size sampling by using a census listing for each village. In the second stage, children were randomly selected from the village census list until the required number of children per village was selected (maximum n = 37). Eligibility criteria were as follows: apparently healthy children aged 6 to 59 mo with no detectable medical reasons for poor health or chronic disease and whose primary caregivers allowed them to participate. The design sample size per province (n = 854) was sufficient to estimate expected prevalences of deficiency of iron and vitamin A and genetic Hb disorders by province of 50%, 20%, and 35%, respectively, with a precision of 5% with 95% confidence, allowing for 10% attrition and a design effect of 2.0 (14). Ethical approval for the study was granted by the Cambodian National Ethics Committee for Health Research. Written informed consent was obtained from the parents or caregivers of each child.

Assessment of child health status, household characteristics, and anthropometric measurements. Trained Cambodian field workers administered a pretested structured questionnaire to the parents or caregivers in their homes, collecting information on the index child including age, sex, episodes of recent illness (acute respiratory infection, diarrhea, and fever), and the administration of deworming tablets (mebendazole) and iron and vitamin A supplements. Household characteristics were also recorded, including maternal education, mortality rates for infants and children <5 y old, and household assets.

Weight and recumbent length or height (for children aged ≥2 y) were measured for each child by trained anthropometrists with children wearing light clothing and no shoes. Measurements were taken by using standardized techniques and calibrated equipment (15) in duplicate; a third measurement was taken if the difference between the first 2 measurements was outside the allowable difference for that measure (16). Height-for-age, weight-for-age, and weight-for-height Z-scores were calculated for the children aged 6 to 60 mo from the WHO 2007 multicenter growth reference data by using the computer program WHO Anthro 2007 (17). Extreme Z-scores were excluded from the data set (18). Stunting, underweight, and wasting were defined by height-for-age, weight-for-age, and weight-for-height Z-scores of < -2 SD, respectively, below the mean or median values of the WHO growth reference data.

Laboratory assessment. Blood from nonfasting morning or early afternoon venipuncture was collected into separate evacuated tubes (Becton Dickinson) either with or without EDTA as an anticoagulant. It was kept cold before processing in the NIPHL, Phnom Penh, Kampong Thom, and Battamang provincial hospital laboratories. In all cases a complete blood count (including RDW) was performed by using an automated hematology analyzer (Sysmex KX21, Sysmex Corp.).

After separation, serum samples were frozen in polyethylene vials at −70°C for later analysis for ferritin, sTfR, RBP, AGP, and CRP using an ELISA (19) in the laboratory of SEAMEO, Regional Center for Community Nutrition, University of Indonesia, Jakarta, Indonesia.

Hb genotyping was conducted on EDTA anticoagulated whole blood. Some children with a mean cell volume >79 fL (n = 154) were excluded from the Hb genotyping because they were unlikely to have one of the genetic Hb disorders known to occur in Cambodia (6), whereas for 225 children there was either an insufficient blood sample or errors in the assay. The NIPHL, Cambodia, analyzed 262 samples by using PCR procedures from the ViennaLab Diagnostics GmbH, Vienna, Austria, that covered 21 α-globin and 22 β-globin mutations (20). Genotyping for the remaining participants was carried out by PCR at the Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Thailand. In this article we include only data for the participants with a normal Hb type (Hb AA) and for the 4 Hb variants for which the number of children with each variant was ≥100. The 4 Hb variants included were as follows: Hb E trait (Hb AE), Hb E homozygote (Hb EE), α^+-thalassemia trait (i.e., deletion of 1 of the 2 α-globin genes), and Hb E trait with α^+-thalassemia trait (i.e., structural defect in one of the β-globin chains plus a deletion of 1 of the 2 α-globin genes). Children with these 2 abnormal Hb types were classified with thalassemia minor (asymptomatic α-thalassemia).

The WHO definition of anemia (Hb <110 g/L) was used (21). Both serum ferritin and sTfR were influenced by the genetic Hb disorders. Hence, IDA and storage iron depletion were defined in the Hb AA children only by low serum ferritin (i.e., <12 μg/L), corrected for subclinical inflammation (22), with or without anemia, as recommended by WHO/CDC (23). Tissue iron deficiency was defined by an sTfR >8.3 mg/L (19). For CRP and AGP, serum concentrations >5 mg/L and >1 g/L were taken to indicate acute and chronic inflammation, respectively (22).

Malaria screening was performed on thick and thin blood films stained with 10% buffered Giemsa solution and examined with a 100× oil immersion objective by experienced technicians from the NIPHL (24).

Stool collection containers were distributed to the caregivers of the participants who were instructed to return the stool samples on the following day, at which time they were transported on ice to the NIPHL. A single stool sample was obtained from 1880 children. Standard WHO (25) procedures were followed to determine the frequency of intestinal parasite infestation in the stool sample by using 0.85% sodium chloride and 0.5% Lugol iodine for the detection of ova, trophozoites, and cysts. One slide was examined per stool.

Statistical analysis. Age was not treated as a continuous variable because relationships between age and many of the biomarkers were nonlinear. Instead, the children were divided into 3 age groups: 6.0–11.99 mo, 12–23.99 mo, and 24–59.99 mo. Serum ferritin, sTfR, and RBP were log transformed before analysis to better approximate a normal distribution. Contingency tables of sociodemographic status; household assets and characteristics; stunting, underweight, and wasting; health status; and morbidity variables by rural versus urban settings were tested by using Pearson's chi-square test. For children aged 24 to 59.99 mo, the means and SD of hematologic variables and biomarkers of iron and vitamin A status for the Hb AA group were compared with each of the 4 major abnormal Hb variants by using the adjusted Wald test, after correcting serum ferritin and RBP for subclinical infection (22,26). Means (±SD) of these same biomarkers for the Hb AA children were calculated by setting, sex, and age group, and differences were assessed by regression analyses.

Multiple linear regression analyses were performed to identify the independent predictors of Hb, log ferritin, log sTfR, and log RBP. The explanatory variables included in the regression models were those that were known (8) or suspected (27) to be biologically important. Indicator variables were used for infection (CRP >5 mg/L), age group (n = 3), genetic Hb types (n = 5), and setting (rural vs. urban). There was no evidence in the multiple regression models of significant multiple collinearity among the independent variables. Logistic regression analysis was also used to calculate the OR and 95% CI for children with Hb <110 g/L, which is indicative of anemia.

Differences in the prevalence of storage iron depletion, tissue iron deficiency, IDA and anemia, and vitamin A deficiency by setting were assessed in the Hb AA children by using contingency table analyses and Pearson's chi-square test for these categorical variables. All P values were 2-sided and were not adjusted for multiple testing. Differences were considered significant at $P < 0.05$. All of the statistical analyses were performed by using STATA version 11 (Stata Corporation), accounting for the survey design with 4 strata (provinces) and 108 primary sampling units (villages).

Results

Sociodemographic status, household characteristics, child morbidity, and health and anthropometric status. A total of 3416 eligible children were recruited for the study; blood for a complete blood cell count was obtained from 2695 children. Hb genotyping was performed on the blood samples from 2316 children (**Supplemental Fig.1**). The proportion of males to females and the age distribution were similar in the group of 3 rural provinces relative to Phnom Penh. However, there were some striking differences in maternal education, mortality rates for children aged <5 y, and household variables between "rural" and "urban" settings (**Table 1**).

Overall, the prevalence of stunting and underweight was significantly higher in the rural compared with the urban children (**Table 2**), and more prevalent among boys (stunting: 39.2 vs. 34.5% in males vs. females, respectively; P = 0.021; underweight: 33.7 vs. 29.2% in males vs. females, respectively; P = 0.008). The prevalence of wasting overall was much lower (12%) compared with stunting and was greater in urban compared with rural children ($P < 0.022$). Of the children, 5% were both stunted and wasted.

In general, infection rates were higher in rural compared with urban children (Table 2), but the prevalence of intestinal parasites was comparable in the 2 settings and was low for helminths: <1% for hookworm (*Ankylostoma duodeae*), whipworm (*Trichuris trichiura*), and *Hymenolepis nana* and <2% for *Ascaris lumbricoides*. For the intestinal protozoa, overall infection rates were higher, especially for *Giardia lamblia* (25%) and *Entamoeba histolytica* (9%), with slightly higher infection rates for rural versus urban children for *Giardia lamblia* (P = 0.06) and *Entamoeba histolytica* (P = 0.05). For the other intestinal protozoa identified, infection rates were <1%. More children from rural compared with urban households had received deworming medication (mebendazole, 500 mg) in the last 6 mo.

More than 50% of the children in both settings had received a vitamin A supplement within the past 6 mo, whereas very few had received an iron supplement in the past 7 d, especially those in the rural provinces ($P < 0.001$) (Table 2).

Genetic Hb disorders. Hb genotyping results showed that, overall, 42.0% of participants had a normal Hb type (AA), 23.1% had Hb E trait (AE), 14.0% had α^+-thalassemia trait, 8.3% had a combination of Hb E trait with α^+-thalassemia trait, and 5.2% were homozygous for Hb E (EE). The remainder (7.3%) had a range of more unusual Hb variants that occurred in <1% of the population. The prevalence of genetic Hb disorders was higher among the children in the rural compared with the urban setting (60.0 vs. 40.0%; $P < 0.001$). Further details of the genetic Hb disorders of the children will be published elsewhere.

TABLE 1 Sociodemographic status, household assets, and other characteristics of the rural and urban children aged 6 to 59 mo

	Rural		Urban	
	n	%	*n*	%
Sex				
Male	1081	49.9	388	51.0
Female	1087	50.1	372	49.0
Maternal education[1]				
None	518	24.0	79	10.5
Up to level 5	1047	48.5	219	29.2
Level 6 and above	594	27.5	452	60.3
Mother had child that died				
At birth	315	14.5	105	13.9
When <5 y old[1]	422	19.5	63	8.3
Household assets[1]				
Both electricity and cell phone	377	17.4	575	76.0
Either electricity or cell phone	568	26.2	169	22.3
Neither electricity nor cell phone	1222	56.4	13	1.7
Household characteristics				
Household fuel[1]				
Liquid petroleum gas	17	0.8	346	45.8
Coal	130	6.0	172	22.8
Wood	1986	91.6	203	26.8
Other	34	1.6	35	4.6
Flooring[1]				
Earth/clay	67	3.1	24	3.2
Palm/bamboo	1796	82.9	223	29.5
Other	304	14.0	509	67.3
Roof type[1]				
Palm/bamboo	684	31.6	12	1.6
Metal	978	45.1	479	63.4
Tile	384	17.7	100	13.2
Other	121	5.6	165	21.8

[1] Pearson's chi-square test for proportions, $P < 0.001$.

Hematologic, iron, and vitamin A status variables by Hb type. As expected, there were some significant differences in mean hematologic values between the children with a normal Hb type (Hb AA) and those with the 4 abnormal Hb variants (**Table 3**), with mean Hb, MCV, and MCH being significantly less and mean RBC and RDW being significantly greater for the Hb EE group than for the Hb AA group. The geometric mean sTfR was significantly higher for each of the 4 abnormal Hb variants compared with the Hb AA group, whereas those for ferritin and RBP (corrected for subclinical inflammation) were not significantly different across all 5 Hb types (Table 3).

Because some of the abnormal Hb variants modified the hematology and sTfR variables, we examined these markers in children with a normal Hb type (Hb AA, the predominant genotype), assessing differences by age group, setting (rural vs. urban), and sex. Most hematologic variables and iron biomarkers varied significantly by age group (**Supplemental Table 1**). Rural children had significantly lower mean Hb, RDW, ferritin, and RBP but significantly greater mean sTfR than did

TABLE 2 Anthropometric, morbidity, and health characteristics of rural and urban children aged 6 to 59 mo[1]

	Rural		Urban		
	n	%	*n*	%	*P*-value[2]
Stunted (HAZ < −2.0)	881	41.5	183	24.4	<0.001
Underweight (WAZ < −2.0)	731	33.8	186	24.7	0.004
Wasted (WHZ < −2.0)	233	10.8	124	16.5	0.022
Any illness in past 2 wk	1425	65.8	569	75.3	<0.001
Acute respiratory infection in past 2 wk	1337	94.0	515	90.2	0.029
Diarrhea in past 2 wk	314	22.4	148	26.1	0.19
Fever in past 2 wk	384	27.1	131	22.9	0.15
Serum C-reactive protein >5 mg/L	348	17.0	73	11.2	0.003
Serum AGP >1 g/L	832	40.7	167	25.6	<0.001
Anemia (Hb <110g/L)	1189	58.2	213	32.7	<0.001
Retinol binding protein <0.7 μmol/L[3]	73	3.6	12	1.8	0.05
Abnormal genetic Hb disorder	946	60.0	228	40.0	<0.001
Parasitic infection	531	38.6	135	32.2	0.07
Eosinophilia >450/μL	603	27.8	128	16.8	<0.001
Mebendazole given within 6 mo	944	44.6	269	38.0	0.044
Vitamin A supplement within 6 mo	1264	58.9	379	53.5	0.16
Iron supplement given within 7 d	183	8.5	142	18.9	<0.001

[1] AGP, α_1-acid glycoprotein; HAZ, height-for-age Z-score; Hb, hemoglobin; WAZ, weight-for-age Z-score; WHZ, weight-for-height Z-score.
[2] Pearson's chi-square test, $P < 0.05$.
[3] Values corrected for subclinical inflammation.

their urban counterparts ($P < 0.01$). Sex differences for some biomarkers were also apparent (Supplemental Table 1).

Determinants of Hb, serum ferritin, sTfR, RBP, and anemia. In the multiple regression model for Hb (**Table 4**), the 2 youngest age groups and the 4 abnormal Hb variants were all significantly associated with lower Hb concentrations, along with both elevated log sTfR and AGP and a rural setting. Higher log RBP and log ferritin were significantly associated with higher Hb concentrations.

Several of these same factors were also significant determinants of log ferritin (Table 4), although here 3 of the 4 abnormal Hb variants were significantly associated with higher log ferritin. In contrast, the second youngest age group of children (i.e., 12–23.99 mo), log sTfR, and a rural setting were significantly associated with lower log ferritin concentrations.

In the multiple regression analysis for sTfR (Table 4), the 2 youngest age groups and 3 of the 4 abnormal Hb variants, log RBP, elevated AGP, being male, and living in a rural setting, were all significantly associated with higher log sTfR. As expected, log ferritin concentrations were significantly associated with lower log sTfR.

Significant determinants of lower log RBP ($R^2 = 0.13$) were an elevated AGP, living in the rural provinces, and not receiving a vitamin A supplement in the past 6 mo ($P < 0.05$).

Homozygous E (Hb EE) children ($n = 77$) had an 18-fold greater risk of being anemic than did those with a normal Hb type (Hb AA) (**Table 5**): the 3 other abnormal Hb variants were each associated with a smaller but still very significant risk of anemia. Other significant risk factors for anemia included children aged 6 to 11.99 mo and 12 to 23.99 mo and those with a low serum ferritin, an elevated AGP, living in a rural setting, a low RBP, and an elevated sTfR.

Prevalence of anemia, IDA, storage iron depletion, and tissue iron deficiency. Overall, the prevalence of anemia in the Hb AA children was 43%, with a higher prevalence among the rural than the urban children (**Table 6**). Furthermore, anemia prevalence decreased with increasing age (*P*-trend < 0.001) in both settings. These same patterns by setting (and age group) were observed for the prevalence of IDA, tissue iron deficiency, and storage iron depletion in the Hb AA children. The prevalence of vitamin A deficiency was low in both rural and urban Hb AA children (Table 6). In contrast, the corresponding anemia prevalences for children with abnormal Hb variants in the rural and urban provinces were 67% and 43% higher, respectively ($P < 0.001$). Comparison of the prevalence of storage iron depletion, tissue iron deficiency, and IDA between those children with a normal and abnormal Hb type could not be made because some of the abnormal Hb variants modified the iron biomarkers (Tables 3 and 4).

Discussion

This large community-based study emphasizes the complex etiology of childhood anemia in Cambodia and the difficulty of identifying iron deficiency in a setting where both genetic Hb disorders and suboptimal vitamin A status coexist. Most of the genetic Hb disorders are not amenable to intervention, and their

TABLE 3 Hematologic variables, biomarkers of iron status, and RBP for normal Hb type (Hb AA) and the 4 major abnormal Hb variants for children aged 24 to 59 mo[1]

	Hb AA	Hb E trait (Hb AE)	α^+-Thalassemia trait	Hb E trait with α^+-thalassemia trait	Hb E homozygote (Hb EE)
n	531	325	204	122	77
Hb, *g/L*	114 ± 9	110 ± 8*	111 ± 9*	108 ± 8*	98 ± 7*
MCV, *fL*	74.7 ± 3.7	68.5 ± 3.6*	71.3 ± 4.2*	69.8 ± 4.4*	57.3 ± 3.4*
RDW, *%*	13.4 ± 1.2	13.4 ± 2.0	13.7 ± 1.8*	13.7 ± 2.2	19.4 ± 1.9*
MCH, *pg*	25.2 ± 2.0	22.5 ± 1.6*	23.2 ± 2.1*	122.8 ± 2.2*	18.1 ± 1.6*
RBC, 10^{12}/*L*	4.56 ± 0.36	4.90 ± 0.36*	4.82 ± 0.40*	4.77 ± 0.39*	5.42 ± 0.39*
sTfR, *mg/L*	7.1 (5.5, 9.1)	7.4 (5.8, 9.4)*	7.5 (5.6, 10.1)*	7.5 (5.9, 9.6)*	9.6* (7.7, 12.0)
Ferritin[2], μ*g/L*	31.6 (14.9, 67.1)	32.5 (16.1, 65.3)	30.4 (13.8, 67.1)	30.1 (14.4, 62.9)	33.7 (16.5, 68.9)
RBP[2], μ*mol/L*	1.16 (0.89, 1.53)	1.18 (0.93, 1.49)	1.22 (0.94, 1.57)	1.14 (0.88, 1.48)	1.14 (0.89, 1.46)

[1] Values are arithmetic means ± SD or geometric means (−SD, +SD). *Different from Hb AA, $P < 0.05$. Hb, hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; RBC, red blood cell count; RBP, retinol binding protein; RDW, red cell distribution width; sTfR, soluble transferrin receptor.
[2] Values for ferritin and RBP were corrected for subclinical inflammation.

TABLE 4 Multiple linear regression analyses with Hb, log serum ferritin, and log soluble transferrin receptor as dependent variables[1]

	Hb	Log ferritin	Log transferrin receptor
n	2147	2147	2147
R^2	0.51	0.43	0.43
		β Coefficient (95% CI)	
Age group (reference = 24−59.99 mo)			
6−11.99 mo	−7.32 (−8.55, −6.09)**	−0.08 (−0.20, 0.03)	0.17 (0.14, 0.20)**
12−23.99 mo	−2.66 (−3.58, −1.74)**	−0.40 (−0.47, −0.32)**	0.11 (0.08, 0.15)**
Genetic Hb variants (reference = Hb type AA)			
Hb E trait	−3.72 (−4.55, −2.90)**	0.13 (0.06, 0.19)**	0.03 (0.00, 0.05)*
α^+-Thalassemia trait	−2.45 (−3.49, −1.41)**	0.07 (−0.02, 0.17)	0.04 (0.01, 0.07)*
Hb E trait with α^+-thalassemia	−4.36 (−5.58, −3.14)**	0.13 (0.02, 0.24)*	0.03 (−0.01, 0.07)
Hb E homozygous	−11.8 (−13.1, −10.4)**	0.49 (0.37, 0.61)**	0.26 (0.21, 0.31)**
Log ferritin	2.51 (1.95, 3.07)**		−0.17 (−0.18, −0.15)**
Log transferrin receptor	−12.2 (−13.1, −10.4)**	−1.45 (−1.60, −1.29)**	
Log retinol binding protein	5.47 (3.96, 6.97)**	0.48 (0.33, 0.62)**	0.06 (0.00, 0.12)*
AGP >1.0 g/L	− 1.57 (−2.34, −0.79)**	0.58 (0.51, 0.66)**	0.13 (0.10, 0.16)**
Sex, male	−0.13 (−0.83, 0.56)	−0.04 (−0.10, 0.02)	0.05 (0.03, 0.07)**
Setting, rural	−2.20 (−3.49, −0.92)*	−0.10 (−0.18, −0.01)*	0.09 (0.06, 0.12)**

[1] *$P < 0.05$, **$P < 0.001$. AGP, α_1-acid glycoprotein; Hb, hemoglobin.

contribution to anemia is likely to gain increasing importance as conditions in Cambodia improve and anemia control programs become more effective. Here we discussed the multiple determinants of Hb, iron, and vitamin A status and their complex interrelationship.

Determinants of Hb. The 4 common genetic Hb disorders were all major risk factors for anemia (Table 5). Of the tested children with any genetic Hb disorder, 62% had anemia compared with 43% of the remaining Hb AA children ($P < 0.001$). The WHO considers a prevalence of anemia >40% to be a public health concern (21). The anemia observed here was predominantly microcytic hypochromic anemia.

Children in the younger age groups and children with low iron status were at risk of anemia (Tables 4 and 5). Dietary iron intakes were likely to be inadequate (28), especially among the younger children who have higher iron requirements because of their faster growth rates; others have noted similar relationships between age and Hb and iron status (29–31).

Neither helminth infections nor eosinophilia were significant determinants of Hb, even though reported rates of helminthic infections in Cambodia have ranged from ~26% (32) to 54% (12). However, 43% of the children had been treated with deworming medication in the last 6 mo. No malarial parasites were identified in the blood films examined, which is consistent with the low incidence of malaria reported in the provinces studied here (11). In contrast, subclinical chronic inflammation (i.e., elevated AGP) was a risk factor for anemia, presumably through blocking both the release of iron from stores and iron absorption.

Of the children, 58% had received a vitamin A supplement in the previous 6 mo and only 3% had vitamin A deficiency (i.e., RBP <0.7 μmol/L) after correcting RBP for inflammation (26,33,34). Nevertheless, lower RBP concentrations were significantly associated with lower Hb, confirming that even marginal vitamin A status can be associated with low Hb (4,35).

Rural children had significantly lower Hb relative to those from Phnom Penh, which was most likely a reflection of their lower socioeconomic status (Table 1) and iron supplement coverage (Table 2), as noted in some (31) but not all (30) earlier studies.

Biomarkers of iron status. Serum ferritin is the recommended biomarker for detecting iron deficiency, but it is known to be elevated by inflammation (Table 4). In our study, log sTfR was also significantly elevated by chronic inflammation (i.e., elevated AGP) (Table 4), even though sTfR is not generally considered to be influenced by inflammation (23).

Three abnormal genetic Hb disorders were significant positive determinants of log ferritin and log sTfR (Table 4) as noted by others (4,36–38). Elevated ferritin and sTfR concentrations in certain Hb disorders are caused by ineffective erythropoiesis (36,39) that stimulates an increase in dietary iron absorption, even when iron stores are adequate.

The significant positive associations observed here between the iron biomarkers and RBP (Table 4) are almost certainly because of the interaction of vitamin A with iron metabolism, although the precise mechanisms involved are still uncertain (3).

The degree to which the age- and sex-related differences in iron biomarkers (and Hb) (Supplemental Table 1) reflect normal

TABLE 5 Multivariate logistic regression models of risk factors for Hb <110 g/L in children aged 6 to 59 mo (n = 2329)[1]

	OR	(95% CI)	$P >$ |t|
Age group (reference = 24−59.99 mo)			
6−11.99 mo	6.10	(4.28, 8.70)	<0.001
12−23.99 mo	2.71	(2.06, 3.57)	<0.001
Genetic Hb variants (reference = Hb AA)			
Hb E trait	2.29	(1.78, 2.95)	<0.001
α^+-Thalassemia trait	1.42	(1.07, 1.87)	0.015
Hb E trait with α^+-thalassemia	2.67	(1.79, 3.99)	<0.001
Hb E homozygous	18.47	(8.45, 40.37)	<0.001
Ferritin <12 μg/L	3.24	(2.23, 4.71)	<0.001
AGP >1.0 g/L	1.43	(1.19, 1.72)	<0.001
Sex, male	1.15	(0.94, 1.40)	0.17
Setting, rural	2.29	(1.70, 3.09)	<0.001
Retinol binding protein <0.7 μmol/L	3.63	(2.23, 5.90)	<0.001
Soluble transferrin receptor >8.3 mg/L	2.14	(1.73, 2.66)	<0.001

[1] AGP, α_1-acid glycoprotein; Hb, hemoglobin.

TABLE 6 Prevalence of anemia, iron deficiency anemia, tissue iron deficiency, storage iron depletion, and vitamin A deficiency for rural and urban children aged 6 to 59 mo with a normal Hb type (Hb AA)[1]

	Rural			Urban		
Hb AA children	*n/total n*	%	(95% CI)	*n/total n*	%	(95% CI)
Anemia (Hb <110 g/L)	322/631	51.0	(46.2, 55.9)	96/342	28.1*	(22.2, 34.0)
Iron-deficiency anemia (Hb <110 g/L, ferritin[2] <12 μg/L)	125/631	21.4	(17.8, 25.0)	32/234	9.4*	(6.2, 12.5)
Tissue iron deficiency (sTfR >8.3 mg/L)	292/630	46.3	(41.3, 51.4)	81/341	23.8*	(18.7, 28.8)
Storage iron depletion (ferritin[2] <12 μg/L)	169/631	26.8	(22.9, 30.7)	53/342	15.5*	(11.8, 19.2)
Vitamin A deficiency (RBP[2] <0.7 μmol/L)	28/631	4.4	(2.6, 6.3)	7/342	2.0	(0.4, 3.7)

[1] Values are % (95% CI). *Different from rural, $P < 0.001$. Hb, hemoglobin; RBP, retinol binding protein; sTfR, soluble transferrin receptor.
[2] Values for serum ferritin and RBP were corrected for subclinical inflammation.

physiologic changes during infancy and early childhood rather than diet-associated changes in iron status is uncertain. Lower iron status for younger (i.e., <24 mo) compared with older children is perhaps associated, at least in part, with failure to supply sufficient readily available iron from nonmilk foods in late infancy (7,8,29). From age 24 to 36 mo, Cambodian toddlers are often fed rice porridge enriched with pork blood (28), a practice that may have contributed to the better iron status of the older children.

Genetic differences (40) or the higher tissue iron requirements associated with the more rapid growth rate of males (41) may have led to the higher sTfR (Table 4) concentrations in boys than in girls. Requirements for growth-limiting micronutrients such as zinc are also greater in males (8), and hence may account for the higher prevalence of stunting and underweight reported here in boys.

Differences in maternal education, socioeconomic status, coverage of iron supplementation, and prevalence of subclinical inflammation may have contributed to the poorer iron status of the rural versus the urban children (Table 4).

Strengths and weaknesses of the study. To our knowledge, this is the first community-based study to characterize 4 common genetic Hb disorders in a large group of Cambodian children using appropriate DNA analysis, and to highlight the uncertainties of using ferritin and sTfR to assess the prevalence of storage and tissue iron deficiency in such settings.

Our cross-sectional survey does not permit causal inferences to be made from reported associations. Furthermore, the limitations in the selection of the children and the blood samples for the assay of the genetic Hb disorders preclude the extrapolation to all children aged 6 to 59 mo in the four provinces or nationally. Nevertheless, several of the nutritional and household variables for the rural children were comparable to those reported for rural children aged <5 y in the CDHS (1), including the following: prevalence of anemia, stunting, and underweight; maternal educational attainment; and household characteristics (Table 1). Sociodemographic status, household assets, and maternal education, however, were slightly higher for our Phnom Penh sample compared with the urban CDHS sample (1).

Notwithstanding their wide age range (i.e., 6 to 59 mo), we used the WHO Hb cutoff for anemia (21), facilitating comparison with the CDHS data (1). However, several studies indicated that the use of this single cutoff (110 g/L) is inappropriate. Lower cutoffs of 100 g/L (42) or 105 g/L (43) have been proposed for infants and young children.

Results for ferritin and sTfR were confounded by genetic Hb disorders and vitamin A status. Clearly, before valid national prevalence estimates of IDA, storage iron depletion, and tissue iron deficiency can be determined in Cambodia, adjustments for the influence of genetic Hb disorders and low vitamin A status on ferritin and sTfR are essential, as noted earlier for schoolchildren in northeast Thailand (4).

Finally, because of the difficulties in generating prevalence estimates for iron deficiency, we restricted our estimates of IDA, storage iron depletion, and tissue iron deficiency to those children with normal Hb type (Hb AA). However, it is likely that the prevalence of nutritional iron deficiency among the children with abnormal genetic Hb disorders will be comparable to those with Hb type AA.

In summary, our results emphasize the high prevalence of anemia among these Cambodian children aged 6 to 59 mo and the difficulties of identifying iron deficiency in the presence of genetic Hb disorders. Important risk factors for anemia were a genetic Hb disorder (most notably Hb EE), age <2 y, low vitamin A status, storage iron depletion, living in a rural setting, tissue iron deficiency, and subclinical chronic inflammation. Most of the genetic Hb disorders are not amenable to intervention. Hence, for maximum effectiveness, programs to prevent anemia in Cambodia should focus on children 6 to 23 mo of age from rural households and should aim to increase vitamin A supplementation coverage, reduce infections, and enhance both the amount and quality of complementary foods through nutrition education, dietary diversification and modification, and micronutrient fortification. Clearly, in Cambodia as well as other resource-poor countries in South East Asia, low-cost methods for detecting genetic Hb disorders are urgently required.

Acknowledgments

This study was undertaken as a partnership between World Vision, the Cambodian National Nutrition Program, and the National Institute of Public Health, Phnom Penh. The authors acknowledge the following individuals for their contribution to this joint effort: Dr. Nhek Bunchhup, Director, Battambang Provincial Health Department; Dr. Va Lungkhun, Director, Kampong Thom Provincial Health Department; Dr. Koy Bunphanny, Director, Preah Vihear Provincial Health Department; Dr. Sok Sokun, Director, Phnom Penh Provincial Health Department; Dr. Nigot Sithy, Battambang Provincial Hospital; Dr. Srey Sin, Kampong Thum Provincial Hospital; and Dr. Juergen Erhardt, SEAMEO-TROPMED, Regional Center for Community Nutrition, University of Indonesia, Jakarta, Indonesia. J. G., M. Y., B. M., R. D., and U. S. A. designed the research project, developed the overall research plan, and provided study oversight; J. G., M. Y., and R. D. conducted the study; C. A., R. S. G., and S. M. W. analyzed the data; R. S. G.

wrote the initial draft of the manuscript; and J. G., M. Y., S. M. W., and R. S. G. shared responsibility for the final content. All authors read and approved the final manuscript.

Literature Cited

1. Cambodia Demographic and Health Survey. Cambodia Demographic and Health Survey 2005. Phnom Penh, Cambodia, and Calverton, MD: National Institute of Statistics, Directorate General for Health and ORC Macro; 2006.
2. Helen Keller International. Iron deficiency in Cambodia: the need for iron supplementation among preschool-aged children. Cambodian Nutr Bull. 2001;2:1–4.
3. West KP, Gernand AD, Sommer A. Vitamin A in nutritional anemia. In: Kraemer K, Zimmermann MB, editors. Nutritional anemia. Basel (Switzerland): Sight and Life Press; 2007.
4. Thurlow RA, Winichagoon P, Green T, Wasantwisut E, Pongcharoen T, Bailey KB, Wasantwisut E, Gibson RS. Only a small proportion of anemia in North East Thai school children is associated with iron deficiency. Am J Clin Nutr. 2005;82:380–7.
5. Helen Keller International. National Institute of Public Health, Phnom Penh, Cambodia. Cambodia Micronutrient Survey. Cambodia Nutr Bull. October 2000;2:5–8.
6. Carnley BP, Prior JF, Gilbert A, Lim E, Devenish R, Sing H, Sarin E, Guhadasan R, Sullivan S, Wise CA, et al. The prevalence and molecular basis of hemoglobinopathies in Cambodia. Hemoglobin. 2006;30:463–70.
7. Giovannini M, Sala D, Usuelli M, Livio L, Francescato G, Braga M, Radaelli G, Riva E. Double-blind, placebo-controlled trial comparing effects of supplementation with two different combinations of micronutrients delivered as sprinkles on growth, anemia, and iron deficiency in Cambodian infants. J Pediatr Gastroenterol Nutr. 2006;42:306–12.
8. Anderson VP, Jack S, Monchy D, Hem N, Hok P, Bailey KB, Gibson RS. Co-existing micronutrient deficiencies among stunted Cambodian infants and toddlers. Asia Pac J Clin Nutr. 2008;17:72–9.
9. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ. 2001;79:704–12.
10. Weatherall DJ. Thalassemia as a global health problem: recent progress toward its control in the developing countries. Ann N Y Acad Sci. 2010;1202:17–23.
11. WHO. Malaria in the greater Mekong subregion: regional and country profiles, 2010. http://www.wpro.who.int/internet/resources.ashx/MVP/docs/MAL-260.pdf.
12. Lee K-J, Bae Y-T, Deung Y-K, Ryang Y-S, Kim H-J, Im K-I, Young T-S. Status of intestinal parasite infections among primary school children in Kampongcham, Cambodia. Korean J Parasitol. 2002;40:153–5.
13. Koski KG, Scott ME. Gastrointestinal nematodes, nutrition and immunity: breaking the negative spiral. Annu Rev Nutr. 2001;21:297–321.
14. Gorstein J, Sullivan KM, Parvanta I, Begin F. Indicators and methods for cross-sectional surveys of vitamin and mineral status of populations. Atlanta (GA): Micronutrient Initiative (Ottawa) and the Centers for Disease Control; 2007.
15. WHO. Anthropometry training video: the WHO multicentre growth reference study (DVD version). The WHO Multicentre Growth Reference Study. Geneva: WHO; 2004.
16. de Onis M, Onyango AW, Van den Broeck J, Chumlea WC, Martorell R. Measurement and standardization protocols for anthropometry used in the construction of a new international growth reference. Food Nutr Bull. 2004; 25(1 Suppl):S27–36.
17. Blössner M, Siyam A, Borghi E, de Onis M, Onyango A, Yang H. WHO Anthro for Personal Computers: software for assessing growth and development of the world's children. Geneva: Department of Nutrition for Health and Development, WHO; 2007.
18. WHO. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. Geneva: WHO; 1995.
19. Erhardt JG, Estes JE, Pfeiffer CM, Biesaiski 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.
20. Puehringer H, Najmabadi H, Law H, Krugluger W, Viprakasit V, Pissard S, Baysal E, Taher A, Farra C, Al-Ali A, et al. Validation of a reverse-hybridization StripAssay for the simultaneous analysis of common a-thalassemia point mutations and deletions. Clin Chem Lab Med. 2007;45:605–10.
21. WHO. Iron deficiency anaemia: assessment, prevention, and control. A guide for programme managers. Geneva: UNICEF/UNU/WHO; 2001.
22. 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.
23. WHO/CDC. Assessing the iron status of populations. Geneva: Department of Nutrition for Health and Development, WHO; 2005.
24. Moody A. Rapid diagnostic tools for malaria parasites. Clin Microbiol Rev. 2002;15:66–78.
25. WHO. Manual of basic techniques for a health laboratory. 2nd ed. Geneva: WHO; 2003.
26. Thurnham DI, McCabe GP, Northrop-Clewes C, Nestle P. Effects of subclinical infection on plasma retinol concentrations and assessment of prevalence of vitamin A deficiency: meta-analysis. Lancet. 2003;362:2052–8.
27. Van dePoel E, Hosseinpoor AR, Speybroeck N, Van Ourti I, Vega J. Socioeconomic inequality in malnutrition in developing countries. Bull World Health Organ. 2008;86:282–91.
28. Anderson VP, Cornwall J, Jack S, Gibson RS. Intakes from non-breastmilk foods for stunted toddlers living in poor urban villages of Phnom Penh, Cambodia. Matern Child Nutr. 2008;4:146–59.
29. Aggett PJ, Agostoni C, Axelsson I, Bresson JL, Goulet O, Hernell O, Koletzko B, Lafeber HL, Michaelsen KF, Micheli JL, et al. Iron metabolism and requirements in early childhood: do we know enough? a commentary by the ESPGHAN Committee on Nutrition. J Pediatr Gastroenterol Nutr. 2002;34:337–45.
30. Agho KE, Dibley MJ, D'Este C, Gibberd R. Factors associated with haemoglobin concentrations among Timor-Leste children aged 6–59 months. J Health Popul Nutr. 2008;26:200–9.
31. Osório MM, Lira PIC, Ashworth A. Factors associated with Hb concentration in children aged 6–59 months in the State of Pernambuco, Brazil. Br J Nutr. 2004;91:307–15.
32. Park SK, Kim D-H, Deung Y-K, Kim H-J, Yang E-J, Lim S-J, Ryang Y-S, Jin D, Lee K-J. Status of intestinal parasite infections among children in Bat Dambang, Cambodia. Korean J Parasitol. 2004;42:201–3.
33. de Pee S, Dary O. Biochemical indicators of vitamin A deficiency: serum retinol and serum retinol binding protein. J Nutr. 2002;132:2895S–901S.
34. Hix J, Rasca P, Morgan J, Denna S, Panagides D, Tam M, Shankar AH. Validation of a rapid enzyme immunoassay for the quantitation of retinol-binding protein to assess vitamin A status within populations. Eur J Clin Nutr. 2006;60:1299–303.
35. Ahmed F, Khan M, Karim R, Taj S, Hyderi T, Faruque MO, Margetts BM, Jackson AA. Serum retinol and biochemical measures of iron status in adolescent school girls in urban Bangladesh. Eur J Clin Nutr. 1996;50:346–51.
36. Rees DC, Williams TN, Maitland K, Clegg JB, Weatherall DJ. Alpha thalassaemia is associated with increased soluble transferrin receptor levels. Br J Haematol. 1998;103:365–9.
37. Ong KH, Tan HL, Tam LP, Hawkins CW, Kuperan P. Accuracy of serum transferrin receptor levels in the diagnosis of iron deficiency among hospital patients in a population with a high prevalence of thalassaemia trait. Int J Lab Hematol. 2008;30:487–93.
38. 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.
39. Worwood M. Serum transferrin receptor assays and their application. Ann Clin Biochem. 2002;39:221–30.
40. Domellöf M, Lönnerdal B, Dewy KG, Cohen RJ, Rivera LL, Hernell O. Sex differences in iron status during infancy. Pediatrics. 2002;110:545–52.
41. Wieringa FT, Berger J, Dijkhuizen MA, Hidayat A, Ninh NX, Utomo B, Wasantwisut E, Winichagoon P. Sex differences in prevalence of anemia and iron deficiency in infancy in a large multi-centre trial in South-East Asia. Br J Nutr. 2007;98:1070–6.
42. Sherriff A, Emond A, Hawkins N, Golding J;. ALSPA Children in Focus Study Team. Haemoglobin and ferritin concentrations in children aged 12 and 18 months. Arch Dis Child. 1999;80:153–7.
43. Michaelsen KF, Milmann N, Samuelson G. A longitudinal study of iron status in healthy Danish infants: effects of early iron status, growth velocity and dietary factors. Acta Paediatr. 1995;84:1035–44.