Comparability of Inflammation-Adjusted Vitamin A Deficiency Estimates and Variance in Retinol Explained by C-Reactive Protein and α_1 -Acid Glycoprotein during Low and High Malaria Transmission Seasons in Rural Zambian Children

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Abstract. Inflammation-induced hyporetinolemia (IIH), a reduction in serum retinol (SR) during inflammation, may bias population estimates of vitamin A deficiency (VAD). The optimal adjustment for IIH depends on the type and extent of inflammation. In rural Zambian children (4–8 years, $N = 886$), we compared three models for defining inflammation: α_1 -acid glycoprotein (AGP) only (inflammation present if > 1 g/L or normal if otherwise), C-reactive protein (CRP) only (moderate inflammation, 5–15 mg/L; high inflammation, > 15 mg/L; or normal if otherwise) and a combined model using both AGP and CRP to delineate stages of infectious episode. Models were compared with respect to 1) the variance in SR explained and 2) comparability of inflammation-adjusted VAD estimated in low and high malaria seasons. Linear regression was used to estimate the variance in SR explained by each model and in estimating the adjustment factors used in generating adjusted VAD (retinol < 0.7 $\mu\text{mol/L}$). The variance in SR explained were 2% (AGP-only), 11% (CRP-only), and 11% (AGP–CRP) in the low malaria season; and 2% (AGP-only), 15% (CRP-only), and 12% (AGP–CRP) in the high malaria season. Adjusted VAD estimates in the low and high malaria seasons differed significantly for the AGP (8.2 versus 13.1%) and combined (5.5 versus 9.1%) models but not the CRP-only model (6.1 versus 6.3%). In the multivariate regression, a decline in SR was observed with rising CRP (but not AGP), in both malaria seasons (slope = -0.06 ; $P < 0.001$). In this malaria endemic setting, CRP alone, as opposed to CRP and AGP, emerged as the most appropriate model for quantifying IIH.

BACKGROUND

Childhood vitamin A deficiency (VAD) is a serious public health problem, with adverse consequences for morbidity and mortality.¹ It is estimated that up to 202 million preschool children globally may be deficient, defined as having a serum (or plasma) retinol concentration below 0.7 $\mu\text{mol/L}$.² The prevalence of VAD is highest in the World Health Organization (WHO) African region, where children are unable to meet the requirements for rapid growth owing to inadequate dietary intake and increased losses from prevalent infections.^{3–5} In these settings, there remains a need for continued monitoring of VAD to inform the design of appropriate interventions. The assessment of vitamin A status is, however, problematic particularly in regions with a high prevalence of infections. It has been shown that concentrations of serum retinol (SR), the commonly used biomarker of vitamin A status,⁶ are reduced during the acute phase response to infections.^{7–10} This phenomenon, referred to as inflammation-induced hyporetinolemia (IIH),^{11–14} is not completely understood and may lead to misclassification of vitamin A status at the child and population levels.

It is the recommendation of the WHO that to guide the interpretation of serum or plasma retinol concentrations, the potential influence of inflammation be characterized by concurrently assessing one or more acute phase proteins (APPs).¹⁵ Although methods for the adjustment of IIH are evolving, α_1 -acid glycoprotein (AGP) and C-reactive protein (CRP) remain the most commonly used APPs. Furthermore, the practice of using both AGP and CRP to control for IIH is

becoming conventional.^{15–17} This approach, originally proposed by Thurnham et al.,¹⁷ is based on two important assumptions, namely 1) the inflammation-associated reduction in retinol coincides with the sequential rise in both CRP (during the early phase) and AGP (during the late phase), and 2) the early and late phases of inflammation likely affect retinol concentrations differently. Thus, adjustment for both APPs may be necessary to fully account for IIH. A limitation of this approach is the potential for over-adjustment. This method inherently assumes that any observed difference in retinol, between children with and without inflammation, is entirely attributable to the inflammation and is transient, and can therefore be mathematically corrected to reflect the distribution of values that would have occurred in the absence of inflammation. However, the necessity or appropriateness of universally adjusting retinol concentrations among individuals in the late stages of inflammation, where AGP (but not CRP) is elevated, remains debatable.^{17,18} In a systematic review by Thurnham et al.,¹⁷ the ratio of plasma retinol concentration among children in the late convalescent stage (elevated AGP but normal CRP) to that of apparently healthy individuals was estimated to be 0.94 – 1.34 , suggesting that elevated AGP is not always associated with a reduction in retinol. A recent study by Wessells et al.,¹⁸ which reported that the concentration of retinol binding protein (RBP), a proxy indicator for SR, was about 18% higher in children with elevated AGP (but normal CRP), compared with children with normal levels of both APPs, further raises questions about the consistency of the association between AGP and retinol, and hence, its utility in quantifying IIH. Application of adjustment factors in this context, where AGP is positively (as opposed to negatively) correlated with retinol or its binding protein would be inconsistent with our current understanding of the changes in retinol over the course of the acute phase reaction.^{17,19,20}

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Because of this potential for over-adjustment, and also considering the cost of running laboratory assays for both CRP and AGP, it is critical that the APPs be assessed only if there is strong evidence of their relevance in characterizing population vitamin A status. Ideally, any model for adjusting IHH should meet the following minimum criteria: 1) the variance in retinol explained by the model should increase when the intensity of the infection or inflammation is increased; and 2) the model should produce a consistent and reliable estimate of VAD when applied at different time points in the same, non-intervened population. In this paper, we explored the extent to which AGP and CRP meet these minimum criteria across two malaria seasons (low and high transmission) among children in rural Zambia. In light of new evidence suggesting that malaria may account for additional variance in retinol, beyond that explained by the conventional APPs,^{18,21,22} a secondary aim was to determine whether the inclusion of malaria in the optimal correction model further changes the magnitude of the VAD estimates.

MATERIALS AND METHODS

Ethical clearance. Ethical approval was obtained from the Institutional Review Board of the Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, and the Ethics Review Committee of the Tropical Diseases Research Center (TDRC), Ndola, Zambia.

Subjects and sample collection. This study included children 4–8 years of age from rural Mkushi District, a malaria endemic setting in Zambia. Data for this analysis were collected as part of baseline and endline assessments during the implementation of a cluster-randomized controlled trial designed to evaluate the impact of provitamin A carotenoid biofortified maize meal consumption on vitamin A status in this population of preschool-aged children (registered as NCT01696148 at www.clinicaltrials.gov). Detailed methods for the parent study have been published elsewhere.²³ We obtained consent from a parent or legal guardian at the time of the baseline assessment (September 2012), which coincided with the low malaria transmission season and collected data on the history of morbidity, dietary intake, and socioeconomic status. At a central site, we measured height with a Shorr board to the nearest 0.1 cm, weight with a SECA 874 digital scale to the nearest 0.1 kg, mid-upper arm circumference with insertion tapes to the nearest 0.1 cm and tricipital skinfold with a Holtain caliper to the nearest 0.1 mm. Axillary temperature was taken with a digital thermometer, and children with high fever (axillary temperature > 39°C) were referred to the nearest health center. From each child, trained laboratory technicians collected approximately 7 mL of venous blood into blood collection tubes (Covidien Monoject sterile tubes with no additives). Malaria diagnosis was done in the field using a rapid diagnostic test (RDT; SD Bioline Malaria Ag P.f, Standard Diagnostics, Yongin, South Korea; 05FK50). Children testing positive were treated with Coartem in accordance with Zambian national guidelines.²⁴ Hemoglobin was assessed using a Hemocue Hb 201 + hemoglobinometer (Angelholm, Sweden). In addition, thick and thin venous blood films were prepared using ~2 and 10 µL of blood, respectively. Whole blood was transported in cooler boxes containing ice packs to the field laboratory for processing. Samples were centrifuged for 10 minutes under dim light. Serum was aliquoted into pre-labeled

cryovials and transported in liquid nitrogen to TDRC for storage at –80°C until analyzed. Data collection procedures were repeated in the same children at the endline assessment, which coincided with the high malaria transmission season (March 2013).

Laboratory analyses. A reversed-phase HPLC procedure was used in the determination of baseline and endline SR concentration. A commercial enzyme-linked immunosorbent assays kit was used to determine serum concentrations of AGP (AGP; Abcam, Cambridge, MA; catalog # ab108854). CRP was measured on an Immulite analyzer (Immulite 1000; Siemens Medical Solutions Diagnostics, Malvern, PA; LKCRP1). Malaria slides, stained with 3% Giemsa, were washed, dried, and read independently by two technicians using light microscopy. Whenever necessary, a third independent reading was done to resolve discordant pairs. For each positive slide, the corresponding thin film was read to determine the *Plasmodium* species. Laboratory procedures were carried out by Craft Technologies (SR), the Johns Hopkins Bloomberg School of Public Health (CRP) and TDRC (AGP and malaria slides).

Definitions. The definition of inflammation was model-dependent. In the univariate AGP model, two inflammation categories were defined, namely reference (AGP ≤ 1 g/L) or inflammation (AGP > 1 g/L). In the univariate CRP model, we defined three categories of inflammation, namely reference (CRP < 5 mg/mL), moderate (CRP 5–15 mg/mL), and high (CRP > 15 mg/mL). In the reference model, four categories of inflammation were defined, namely reference (AGP ≤ 1 g/L and CRP ≤ 5 mg/L), incubation (AGP ≤ 1 g/L and CRP > 5 mg/L), early convalescence (AGP > 1 g/L and CRP > 5 mg/L), and late convalescence (AGP > 1 g/L and CRP ≤ 5 mg/L). VAD was defined as SR concentration < 0.7 µmol/L. Children were considered to have malaria if they had *Plasmodium falciparum* parasitemia of any density as defined by either microscopy or RDT or both, and malaria negative if both RDT and microscopy were negative. This combined malaria definition was implemented because we observed that both RDT- and microscopy-defined malaria were associated with significant elevations in both ferritin and soluble transferrin receptor (sTfR). To characterize the baseline status of the study population, we defined VAD as retinol < 0.7 µmol/L and iron deficiency as ferritin < 12 µg/L in children < 5 years and ferritin < 15 µg/L in older children, as previously described.²⁵ Anemia was defined as hemoglobin < 110 g/L for children < 60 months and < 115 g/L in older children.²⁵ We defined literacy as the ability to read or write in English. We defined stunting and underweight as height-for-age and weight-for-age z-scores, respectively, below –2 standard deviation of the WHO Growth Reference. Fever was defined as axillary temperature > 37.5°C.

Statistical analyses. Data from children who had complete baseline and endline data for SR, AGP, and CRP were included in this analysis. Because the trial intervention did not have an impact on SR concentrations, data were pooled together from both the treatment and control arms of the trial. We conducted sensitivity analyses to further test whether outcomes of interest differed by treatment allocation.

Exploratory analytic techniques including scatter plots, box plots, and kernel density plots were used to examine the individual associations between retinol and CRP or AGP. We explored evidence of linear or nonlinear associations using locally weighted scatter plot smoothing techniques. Skewed distributions, such as CRP, were log-normalized. When the

visual displays showed evidence of a potential change in slope of the association between retinol and either of these two APPs, spline models were constructed to test the statistical significance of the slope change. Subsequently, added variable plots were constructed to examine the association between retinol and CRP or AGP adjusted for the other biomarker. The added variable plot is a visual display of an adjusted regression model, showing the association between an outcome variable (Y) and an explanatory variable (X_1), controlling for interference from one or multiple covariates ($X_2, \dots$). For instance, to examine the association between SR and AGP and controlling for CRP, the slope of the added variable plot, which is a plot of the Y -residual ($Retinol - \beta_0 - \beta_2 CRP$) against the X -residuals ($AGP - \beta_0 - \beta_2 CRP$) represents an approximation of the slope of the regression line $Retinol - \beta_0 - \beta_2 CRP = \beta_1 AGP$. Separate exploratory analyses were performed for the data collected in the low and high malaria seasons.

Based on the exploratory analyses, two regression models, one with AGP (dichotomized) as the only explanatory variable (AGP-only model) and another with CRP (three-groups) as the only explanatory variable (CRP-only model) were constructed. The CRP-only model was informed by our exploratory analyses, which showed a change in slope of the retinol-CRP curve at CRP concentration > 15 mg/L (P of interaction < 0.1). To compare these two models with the Thurnham et al.¹⁷ definition of inflammation, we constructed a third model, the reference model, using both AGP and CRP as described previously. To estimate adjusted VAD prevalence, we first computed model-specific, adjusted retinol concentrations for each child. We estimated group-specific adjustment factors, defined as the difference in mean SR concentrations comparing each inflammatory group within the model to the respective noninflammatory group. For instance, in the AGP-only model, adjustment factors were obtained by subtracting the mean SR concentration of the inflammation group ($AGP > 1$ g/L) from the mean of the reference group ($AGP \leq 1$ g/L). The same approach was applied in estimating the adjustment factors for the moderate and high CRP groups (using a reference of CRP, 5 mg/mL) and for the incubation, early- and late-convalescence groups (using children with normal CRP and normal AGP as the reference). The subtraction of means (instead of ratios) approach was used because the retinol data were normally distributed. To generate adjusted retinol concentrations for the inflammation group, the adjustment factor was added to the measured concentration for each individual within the group. We subsequently estimated adjusted or unadjusted VAD using the adjusted or unadjusted retinol concentrations, respectively. This same approach was applied to the three-group CRP model, and the four-group reference model in estimating the respective model-specific adjusted VAD. For each model, we compared difference in the VAD estimated between the low and high malaria transmission season. Differences in the VAD estimates, whether unadjusted or adjusted for inflammation using the three models were tested using the using McNemar's χ^2 test. In addition, we also estimated the variance in SR concentrations explained by each of the models using regression techniques. Finally, to explore the potential impact of malaria on retinol concentration or VAD estimates, we included a malaria status indicator in the CRP-only (the optimal) model, and subsequently reestimated the concentration of SR in the different inflammation

groups. In this CRP-malaria model, the following six groups were defined: CRP < 5 mg/L without malaria, CRP < 5 mg/L with malaria, CRP < 5 – 15 mg/L without malaria, CRP < 5 – 15 mg/L with malaria, CRP > 15 mg/L without malaria, and CRP < 15 mg/L with malaria. We also reestimated the VAD prevalence adjusted for both CRP and malaria using the same procedures as described previously. Statistical significance was set at $P < 0.05$ except for interaction coefficient, in which case significance was defined as $P < 0.1$. All analyses were conducted with STATA 13 software (StataCorp, College Station, TX).

RESULTS

Data from 886 children who had complete baseline and endline data for SR, AGP, and CRP were included in this analysis. The baseline characteristics of study participants are presented in Table 1. Our study population, which included children aged 68 months on average, is characteristic of a rural, malnourished population with a significant public health problem of both stunting (28%) and anemia (34%). At baseline, nearly one-third of children reported fever in the past 2 weeks, indicating a high prevalence of infection. The subgroup of children included in this analysis were statistically similar to the general population of study participants with respect to age, sex, and nutritional status.

The distributions of SR, inflammation and malaria, in the low and high malaria seasons, are presented in Table 2. A change in malaria prevalence, from 22% in the low transmission season to 51% in the high transmission, was associated with a corresponding increase in inflammation (Table 2), whether

TABLE 1
Baseline socio-demographic characteristics, nutritional status, and morbidity history of study participants

Description	N	Value
Child characteristics		
Age, months	886	68.2 $\pm$ 14.9
Age less than 60 months	886	310 (35.0)
Female (%)	886	440 (49.7)
Nutritional status		
Anemia (%)	884	299 (33.8)
Iron deficiency (%)	877	69 (7.9)
Weight, kg*	863	17.6 $\pm$ 3.2
Height, cm*	862	107.4 $\pm$ 9.3
Stunted†	862	248 (28.0)
Underweight†	862	111 (12.5)
Morbidity history		
Fever in past 2 weeks (%)	873	251 (28.8)
Axillary temperature $> 37.5^\circ\text{C}$	883	7 (0.8)
Cough in past 2 weeks (%)	875	499 (57.0)
Diarrhea in past 2 weeks (%)	874	51 (5.8)
Household characteristics		
Literate household head (%)	852	717 (82.2)
Household with electricity (%)	874	42 (4.8)
Occupation of household head (%)	874	—
Farming/Farm labor	—	235 (26.9)
Self-used	—	241 (27.6)
Salaried worker	—	159 (18.2)

* Arithmetic mean $\pm$ standard deviation.

† Stunting and underweight defined as height-for-age and weight-for-age, respectively, < -2 standard deviations of the WHO Growth Reference (WHO, 2006).^{39,40} Analyses restricted to children who had complete data for serum retinol, α_1 -acid glycoprotein (AGP) and C-reactive protein (CRP) at both baseline and endline ($N = 886$). We defined iron deficiency as ferritin < 12 $\mu\text{g/L}$ in children < 5 years and ferritin < 15 $\mu\text{g/L}$ in older children.²⁵ Anemia was defined as hemoglobin < 110 g/L for children < 60 months and < 115 g/L in older children.²⁵ We defined literacy as the ability to read or write in English. Fever was defined as axillary temperature $> 37.5^\circ\text{C}$.

TABLE 2

Distributions of retinol, malaria, and inflammatory indicators in Zambian children aged 4–8 years in low and high malaria transmission seasons ($N = 744$)

Indicator	Low malaria season (September 2012)	High malaria season (March 2013)	<i>P</i> value
Retinol, $\mu\text{mol/L}$	1.01 ± 0.28	1.00 ± 0.32	0.61
Retinol $< 0.7 \mu\text{mol/L}$ (%)	95 (10.7)	146 (16.5)	< 0.001
Malaria (%)	177 (21.2)	451 (51.0)	< 0.001
Inflammation			
AGP (g/L)	1.14 ± 0.87	1.71 ± 1.01	< 0.001
AGP $> 1.0 \text{ g/L}$ (%)	388 (43.8)	655 (73.9)	< 0.001
CRP, mg/L*	0.79 ± 5.66	2.00 ± 8.67	< 0.001
CRP $> 5.0 \text{ mg/L}$ (%)	146 (16.5)	292 (33.0)	< 0.001

AGP = α_1 -acid glycoprotein; CRP = C-reactive protein.

* Mean (geometric for CRP and arithmetic for AGP and retinol) $\pm$ standard deviation, unless otherwise specified. Statistical test of difference between malaria seasons done with paired *t* test for continuous variables and McNemar's Test for binary variables.

based on AGP (44–74%) or CRP (17–33%). Although mean retinol concentration in the low and high transmission seasons was similar ($\sim 1.0 \mu\text{mol/L}$), the unadjusted VAD prevalence increased from 11% in the low malaria season to 17% in the high malaria season.

Figure 1 shows the scatter plots of the associations between SR concentrations and either AGP or the CRP (log transformed), in the low and high malaria seasons. Overall, retinol concentrations declined with rising CRP concentrations, with an average reduction of about $0.06 \mu\text{mol/L}$ in retinol for every 10 mg/L increase in CRP in both seasons ($P < 0.001$). In both seasons, the CRP-associated decline began at concentrations lower than the 5 mg/L threshold commonly used to define inflammation. In addition, an apparent change in slope of the retinol–CRP curve was observed in the high malaria season, increasing by about 5-fold from a rate of about $0.04 \mu\text{mol/L}$ for CRP values $\leq 15 \text{ mg/L}$ to an average reduction of about $0.2 \mu\text{mol/L}$ for CRP values beyond 15 mg/L for every 10 mg/L increase in CRP (P for interaction < 0.1). Similarly, higher AGP concentrations were associated with lower retinol values, although unlike CRP, the AGP-associated decline was only apparent for concentration beyond the conventional threshold for defining inflammation (1.0 g/L). After adjustment for CRP, however, we found no such negative correlation between AGP and retinol (Figure 2). Rather, we observed in the high malaria season that retinol concentrations appear to increase with rising AGP values. With CRP, however, the negative correlation with retinol persisted even after adjusting for AGP (Figure 2).

Table 3 shows the mean retinol concentrations in apparently normal or inflammation groups as defined by the three models namely AGP-only, CRP-only, and the reference model. In the AGP-only or CRP-only models, the mean retinol concentration in any of the inflammatory categories was lower than the concentration in the respective normal group. Retinol adjustment factors for the various inflammatory groups ranged from a low of $0.05 \mu\text{mol/L}$ for the AGP-only model to a high of $0.31 \mu\text{mol/L}$ for the CRP-only model. In the reference model, retinol concentration in the incubation and early convalescence groups (but not the late convalescence group) were consistently lower than the normal group, with adjustment factors ranging from 0.10 to $0.26 \mu\text{mol/L}$. In the late convalescence group, however, retinol concentrations were slightly higher than the normal population during the high malaria season. In both seasons, the CRP-only model

explained the highest percentage of the variance in retinol, accounting for about 11% and 15% of the variation in retinol in the low and high malaria seasons, respectively. The AGP-only model explained the lowest variance in retinol, accounting for $< 2\%$ of the variation in retinol concentrations in both seasons.

In both seasons, the adjusted VAD estimates were significantly lower than the unadjusted estimate, regardless of the model used (Table 4). Adjustment for inflammation produced different effects on the VAD estimates, depending on the malaria season and the adjustment model used. The adjusted VAD estimated by the CRP-only model was similar (6%) in both the low and high malaria seasons. Adjusted VAD estimated with the AGP and reference models differed significantly between the low and high malaria seasons (Table 4). Of the roughly 11% of children who were classified as VAD using unadjusted retinol concentration in the low malaria season, a significant proportion were subsequently determined to have adequate SR concentrations following adjustment for inflammation with the AGP-only (25%), CRP-only (42%), and reference model (47%). In the high malaria season, where the unadjusted VAD was 17% (Table 4), the proportion of children later determined to have adequate SR concentrations after adjustment were 62% for the CRP-only model, 22% for the AGP-only model, and 45% for the reference model. The results of the sensitivity analyses indicated that the associations of interest were not affected by the interventions delivered by the parent study (Table 4).

The inclusion of malaria to the CRP-only model produced inconsistent effects on the retinol concentrations and adjustment factors for the inflammatory groups (Table 5). In the low malaria season, the inclusion of malaria increased the adjustment factor for the moderate CRP from 0.18 to $0.29 \mu\text{mol/L}$, but decreased the adjustment factor for the high CRP group from 0.34 to $0.27 \mu\text{mol/L}$. The reversed trend was observed in the high malaria seasons, where an increase in adjustment was observed in the high but not the moderate CRP group. Among malaria-positive children who had normal levels of CRP (representation 16% of all children in the low malaria season, and 25% in the high malaria seasons) retinol levels were reduced by $\sim 0.14 \mu\text{mol/L}$ in the low malaria season, but increased by $0.05 \mu\text{mol/L}$ in the high malaria season (Table 5). Although over 50% of malaria cases were associated with elevated CRP (moderate or high) in the high malaria season, only 25% of malaria positives had elevated AGP in the low malaria season. The additional adjustment for malaria reduced VAD prevalence from 6.3% (adjusted for CRP only) to about 3.6% ($P < 0.01$) in the low malaria season, with no significant effect in the high malaria season (Figure 3).

DISCUSSION

The last decade has seen a sustained global interest and increased research efforts toward characterizing the nature and effects of IIH.²⁶ Yet, there is currently no consensus on the optimal model for quantifying the potential bias in VAD prevalence estimates imposed by inflammation. We asserted that an optimal model should 1) explain a greater variance in retinol when the intensity of the inflammation is increased and 2) produce comparable estimates of VAD regardless of the intensity of the inflammation. Here, we evaluated the comparability of three models: an AGP-only model, a CRP-only

FIGURE 1. Scatter plots of serum retinol (SR) against α_1 -acid glycoprotein (AGP) or C-reactive protein (CRP) during low and high malaria transmission seasons among Zambian children ($N = 886$). The left panels show the scatter plots of the associations between SR concentrations and AGP among rural Zambian children in the low (top panel) and high (bottom panel) malaria seasons, respectively. The right panels show the scatter plots of the associations between SR concentration and log-normalized CRP in the low (top panel) and high (bottom panel) malaria seasons, respectively change in slope at CRP concentration of ~ 15 mg/L ($P < 0.1$).

model, and a reference model combining both AGP and CRP. We considered the variance in retinol explained and the adjusted VAD prevalence estimated in the low and high malaria seasons. Based on the aforementioned criteria, we report that in this population of rural Zambia children, the CRP-only model is the most appropriate for addressing inflammation induced hyporetinolemia. Furthermore, we observed that AGP, whether used alone or in combination with CRP, is not ideal, and may in fact, be inappropriate for correcting IIH in regions where malaria is endemic.

Of the three models evaluated, only the CRP model met the two pre-specified criteria for the optimal modeling of IIH. These findings suggest that IIH is largely explained by elevated CRP. In practice, this novel model would be less costly

than having to measure both AGP and CRP. In addition, this model, compared with the recently proposed regression approach, offers a relatively less complex approach to capture the dose-dependent association between SR and the intensity of inflammation. The CRP-only model also has biological plausibility. Evidence from animal models suggests that levels of mRNA for both RBP and transthyretin drop rapidly within the first 36 hours after induction of inflammation,¹⁹ at about the same time that CRP is expected to rise.^{20,27} The proposed three-group classification system used here, although unconventional, was informed by the observed association between retinol and CRP in this population. In fact, had we adopted the conventional dichotomous definition for inflammation (i.e., cut-off of 5 or 10 mg/L), the

FIGURE 2. Added variable plots showing the adjusted associations between serum retinol (SR) and α_1 -acid glycoprotein (AGP) or C-reactive protein (CRP) in the low and high malaria season among rural Zambian children ($N = 886$). The added variable plot (also known as partial regression plot) depicts the association between an outcomes variable (Y) and an explanatory variable (X_1), controlling for interference from another explanatory variable (X_2). The slope of an added variable plot, which is a plot of Y -residual ($Y - \beta_0 - \beta_2 X_2$) against the X -residuals ($X - \beta_0 - \beta_2 X_2$), approximates the slope of the regression line $Y - \beta_0 - \beta_2 X_2 = \beta_1 X_1$. The left panels show the retinol residuals from the regression of SR concentration on AGP and adjusted for CRP, in the low (top panel) and high (bottom panel) malaria seasons, respectively. In the left panel, the y axis is the retinol residuals for the regression of retinol against CRP where the x axis is the AGP residuals for the regression of AGP against CRP. The right panels show the residuals of the regression of SR concentration on CRP and adjusted for AGP, in the low (top panel) and high (bottom panel) malaria seasons, respectively. In the right panel, the y axis is the retinol residuals for the regression of retinol against AGP where the x axis is the CRP residuals for the regression of CRP against AGP. The low and high malaria seasons represent the periods of low malaria prevalence (September 2012) and high malaria prevalence (March 2013), respectively. β = adjusted regression coefficient; P = statistical significance of the regression coefficient.

CRP-only model would still have explained a proportion of the variance in retinol greater than or at least comparable to that explained by the combined AGP-CRP reference model in both the low malaria season (9–10%) and the high malaria season (11–14%). Additional research is needed to understand whether the observed differences in the strength of association between CRP or AGP and retinol is unique to a malaria endemic setting.

Consistent with findings elsewhere,^{28,29} the univariate AGP-only model showed a decline in SR concentrations with rising AGP values, in both malaria seasons. However, this model explained only 2% of the variance in retinol in both malaria seasons despite a substantial increase in the proportion of children with inflammation (from 44% to 74%) across the two seasons. Perhaps of greater relevance, we also

observed that after adjusting for CRP, there was no association between SR and AGP assessed in the low malaria season. Counterintuitively, SR concentrations assessed in the high malaria season appeared to increase with rising AGP concentrations. Similarly, the reference model showed that retinol concentrations increased (rather than decreased) in children who had elevated AGP but normal CRP. This unexpected observation is consistent with findings reported by Wessells et al.¹⁸ among Burkinabe children, where concentrations of RBP was about 18% higher in children in the late convalescent stage relative to an apparently healthy group. The application of adjustment factors to this stage of inflammation, although statistically valid, would require the biologically implausible assumptions that the inflammation triggered an increase in SR. Considering that the mean retinol concentrations in the

TABLE 3

Serum retinol concentrations and adjustment factors for model-specific inflammation groups and variance in retinol explained by three models for correcting inflammation-induced hyporetinolemia in the low and high malaria seasons

Inflammation group	N (%)	Mean ($\mu\text{mol/L}$)	AF ($\mu\text{mol/L}$) ^a	Variance (%)
Low malaria season				
AGP-only model	—	—	—	1.9
Normal (AGP ≤ 1 g/L)	498 (56.2)	1.04 (1.02, 1.06)	—	—
Inflammation (AGP > 1 g/L)	388 (43.8)	0.96 (0.94, 0.99)	-0.08 (-0.04, -0.11)***	—
CRP-only model	—	—	—	10.6
Normal (CRP ≤ 5 mg/L)	746 (83.6)	1.04 (1.03, 1.06)	—	—
Moderate (CRP = 5.1–15 mg/L)	87 (9.8)	0.84 (0.80, 0.89)	-0.20 (-0.14, -0.26)***	—
High (CRP > 15 mg/L)	59 (6.6)	0.75 (0.69, 0.82)	-0.29 (-0.22, -0.36)***	—
Reference model	—	—	—	10.5
Normal (AGP ≤ 1 g/L; CRP ≤ 5 mg/L)	458 (51.3)	1.06 (1.04, 1.08)	—	—
Incubation (AGP ≤ 1 g/L; CRP > 5 mg/L)	42 (4.7)	0.82 (0.74, 0.90)	-0.24 (-0.16, -0.32)***	—
Early convalescence (AGP > 1 g/L; CRP > 5 mg/L)	104 (11.7)	0.81 (0.76, 0.85)	-0.26 (-0.20, -0.31)***	—
Late convalescence (AGP > 1 g/L; CRP ≤ 5 mg/L)	288 (32.3)	1.02 (0.99, 1.06)	-0.04 (0.00, -0.08)	—
High malaria season				
AGP-only model	—	—	—	0.52
Normal (AGP ≤ 1 g/L)	231 (26.1)	1.04 (1.01, 1.07)	—	—
Inflammation (AGP > 1 g/L)	655 (73.9)	0.99 (0.96, 1.01)	-0.05 (-0.01, -0.10)*	—
CRP-only model	—	—	—	15.0
Normal (CRP ≤ 5 mg/L)	599 (67.1)	1.08 (1.06, 1.10)	—	—
Moderate (CRP = 5.1–15 mg/L)	113 (12.7)	0.98 (0.92, 1.04)	-0.10 (-0.04, -0.16)**	—
High (CRP > 15 mg/L)	180 (20.2)	0.76 (0.71, 0.81)	-0.31 (-0.26, -0.36)***	—
Reference model	—	—	—	11.9
Normal (AGP ≤ 1 g/L; CRP ≤ 5 mg/L)	218 (24.6)	1.05 (1.01, 1.08)	—	—
Incubation (AGP ≤ 1 g/L; CRP > 5 mg/L)	13 (1.5)	0.94 (0.75, 1.14)	-0.10 (-0.27, 0.07)	—
Early convalescence (AGP > 1 g/L; CRP > 5 mg/L)	279 (31.5)	0.84 (0.80, 0.88)	-0.20 (-0.15, -0.26)***	—
Late convalescence (AGP > 1 g/L; CRP ≤ 5 mg/L)	376 (42.4)	1.10 (1.07, 1.13)	0.05 (0.00, 0.10)	—

AGP = α 1-acid glycoprotein; CRP = C-reactive protein.

^a Adjustment factor for correcting inflammation induced-hyporetinolemia. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; variance in serum retinol concentrations explained by the model. For the AGP-only model, inflammation was defined as AGP > 1 g/L; in CRP-only model, inflammation was defined as moderate (5–15 mg/L) or high (> 15 mg/L). In the reference model (proposed by Thurnham et al),¹⁷ normal is defined as normal AGP (≤ 1 g/L) and normal CRP (< 5 mg/L); incubation defined as elevated CRP with normal AGP; Early convalescence defined as elevated CRP with elevated AGP; late convalescence = normal CRP with elevated AGP. The low and high malaria seasons represents the periods of low malaria prevalence (September 2012) and high malaria prevalence (March 2013) respectively.

late convalescence stage—1.02 $\mu\text{mol/L}$ in the low malaria season and 1.10 $\mu\text{mol/L}$ in the high malaria season—falls within the normal physiologic range of SR concentrations,³⁰ our findings likely suggests that the rise in AGP may have coincided with the homeostatic mobilization of retinol from hepatic stores in the aftermath of an inflammatory episode.²² Adjusting for retinol in such circumstances would be unnecessary and inappropriate.

There have been suggestions in the literature that the additional assessment of malaria may improve the interpretation of SR and other nutritional biomarkers. Our findings suggest a need for additional adjustment for malaria in the low transmission seasons, but not the high transmission season. The additional adjustment for malaria in the high malaria season did not produce a significant change in the VAD estimate beyond the effect produced by adjusting for CRP alone likely because most malaria cases (52%) were associated with elevated CRP in the high malaria season. Additionally, and perhaps more importantly, malaria was associated with a reduction in retinol only when CRP was concurrently elevated. The acute phase response in malaria is largely an innate mechanism, involving the activation of both CRP-dependent and non CRP-dependent pathways.³¹ It is plausible that when transmission is intense, the CRP-dependent mechanism assumes a more dominant role.³¹ The observed disparity in the association between malaria and CRP across the two malaria seasons may also be explained by the difference in the duration of the acute phase response in the two transmission

seasons. In the low malaria season however, only 25% of malaria cases were associated with elevated CRP, and interestingly, the mean retinol concentration was significantly reduced in all malaria-positive cases, with or without a concurrent elevation in CRP. The immune response to malaria

TABLE 4

Changes in prevalence of vitamin A deficiency after corrections for C-reactive protein and/or α 1-acid glycoprotein in the low and high malaria season

Adjustment model/season*	Vitamin A deficiency (%)		
	All (N = 886)	Pro-vitamin A† (N = 389)	Control‡ (N = 497)
Low malaria season, n (%)			
Unadjusted	95 (10.7)	42 (10.8)	53 (10.7)
CRP-only	54 (6.1)	26 (6.7)	28 (5.6)
AGP-only	73 (8.2)	30 (7.1)	43 (8.7)
Reference	49 (5.5)	21 (5.4)	28 (5.6)
High malaria season, n (%)			
Unadjusted	146 (16.6)‡	63 (16.2)‡	83 (16.7)‡
CRP-only	55 (6.3)	23 (5.9)	32 (6.4)
AGP-only	116 (13.1)‡	52 (13.4)‡	64 (12.9)‡
Reference	81 (9.1)‡	33 (8.5)‡	48 (9.7)‡

AGP = α 1-acid glycoprotein; CRP = C-reactive protein. The numbers represent frequency and proportion of children with vitamin A deficiency. AGP-only model adjusted for AGP; CRP-only model adjusted for CRP; Reference model adjusted for both AGP and CRP as proposed by Thurnham et al.¹⁷ Children in the Pro-vitamin A group received a daily β -carotene biofortified maize meal for 6 months. Control groups did not receive a β -carotene intervention.

* All adjusted estimates significantly lower than corresponding unadjusted estimates in both seasons.

† Estimates were statistically similar between Provitamin A and control groups.

‡ Significantly higher than corresponding estimate in low malaria seasons ($P < 0.01$).

TABLE 5

Serum retinol concentrations among inflammation groups defined by C-reactive protein and malaria and estimated adjustment factors in low and high malaria transmission seasons

Inflammation group ^a	N (%)	Mean retinol (μmol/L)	AF (μmol/L) ^b
Low malaria season (N = 836)			
Normal CRP-no malaria	563 (67.3)	1.07 (1.05, 1.09)	0.00
Moderate CRP-no malaria	58 (6.9)	0.89 (0.83, 0.95)***	-0.18 (-0.11, -0.25)***
High CRP-No malaria	38 (4.6)	0.74 (0.67, 0.80)***	-0.34 (-0.25, -0.42)***
Normal CRP-positive malaria	132 (15.8)	0.94 (0.89, 0.98)***	-0.14 (-0.09, -0.19)***
Moderate CRP-positive malaria	25 (3.0)	0.79 (0.71, 0.86)***	-0.29 (-0.18, -0.39)***
High CRP-positive malaria	20 (2.4)	0.80 (0.65, 0.94)***	-0.27 (-0.16, -0.39)***
High malaria season (N = 885)			
Normal CRP-no malaria	374 (42.3)	1.06 (1.02, 1.08)	0.00
Moderate CRP-no Malaria	40 (4.5)	0.97 (0.86, 1.07)	-0.09 (-0.19, 0.00)
High CRP-no malaria	20 (2.3)	0.88 (0.76, 1.00)	-0.18 (-0.04, -0.31)*
Normal CRP-positive malaria	220 (24.9)	1.11 (1.07, 1.14)	0.05 (0.01, 0.10)*
Moderate CRP-positive malaria	71 (8.0)	0.99 (0.92, 1.07)	-0.07 (-0.14, 0.01)
High CRP-positive malaria	160 (18.1)	0.75 (0.70, 0.80)	-0.31 (-0.25, -0.36)***

AGP = α1-acid glycoprotein; CRP = C-reactive protein; RDT = rapid diagnostic test.

^a CRP defined as normal (< 5 mg/L), moderate (5–15 mg/L) or high (> 15 mg/L). Positive malaria defined as positive microscopy and/or RDT. No malaria defined as negative microscopy and negative RDT.

^b AF = Adjustment factor for correcting inflammation induced-hyporetinolemia; N (%) represent the total number (proportion) of children in each malaria-CRP category *P < 0.05; **P < 0.01 ***P < 0.001; The low and high malaria seasons represents the periods of low malaria prevalence (September 2012) and high malaria prevalence (March 2013) respectively.

involves the initial activations of Th1-type pro-inflammatory pathways for the clearance of parasites, and then later, the activation of Th2-type anti-inflammatory mechanisms for continuing parasite clearance via the adaptive immune mechanisms and downregulation of inflammation.^{32–34} It is plausible that that initial Th1 phase, which involves CRP, is relatively short-lived in the low malaria season, such that CRP levels return to normal, before parasite clearance is complete. In such conditions, the assessment of CRP alone, as opposed to CRP and malaria, may not suffice in characterizing the malaria-related inflammatory changes in retinol.

A multivariate, linear regression approach for adjusting IIH has recently been proposed.^{16,35} The advantage of this approach is that it estimates context-specific adjustment factors, based on observed associations between specific nutritional biomarkers and the acute phase reactants, as opposed to using predefined cut-offs for inflammation. However, this approach has several limitations. First, the universal assumption of linearity is inconsistent with the goal of generating population-specific, data-driven adjustment factors. As

shown in this population, the associations between retinol and the APPs are not always linear. A related problem is the potential to over-adjust by applying the same regression coefficient to every individual. To mitigate the problem of over-adjustment, Larson et al suggested that adjustment factors be applied to individuals at or above the first decile of the log-normalized AGP and CRP concentrations, as opposed to every individual.¹⁶ Had we adopted this approach, we would have adjusted the retinol concentrations in over 90% of our study population. Such a procedure, which would imply that nearly all children have some degree of IIH, may be unjustifiable, even in regions with a high burden of infection. Our data suggest that the issue of over-adjustment may best be addressed by using context-specific thresholds, as opposed to universally adopting the AGP and CRP deciles proposed elsewhere.^{16,35}

A potential limitation of this study is that data were pooled from across clusters of children, some of whom received a provitamin A carotenoid intervention for 6 months. Although the evidence from animal models suggests that a retinol intervention may regulate the inflammatory response,^{35,36} there was no intervention effect on either retinol or inflammation in this population. Our sensitivity analyses confirmed that the intervention did not affect the outcomes of interest in this analysis. Another limitation, which is pertinent to all current adjustment procedures, is the assumption that the observed reductions in retinol are completely transient and that retinol concentrations would return back to normal levels when the inflammation resolves. Unlike other nutritional biomarkers, such as ferritin for instance, which are mainly sequestered, as opposed to excreted, some retinol is lost via increased urinary output during inflammation. Hence in the case of retinol, the resolution of inflammation may not completely correct the IIH. Consequently, the tendency to overadjust is especially great in the case of retinol. Until models are developed to appropriately account for the inflammation-induced urinary losses, retinol adjustment factors must be interpreted cautiously. A major strength of this study is the availability of multiple survey data, which enabled the internal validation of these competing models between two time points with varying burden of inflammation.

FIGURE 3. Changes in vitamin A deficiency (VAD) estimates after correction for C-reactive protein (CRP) alone, or with malaria, in the low and high malaria seasons. The bars show the prevalence of VAD either unadjusted (black), adjusted for CRP alone (gray) or adjusted for both CRP and malaria (white) in the low and high malaria seasons. A = significantly from unadjusted VAD ($P < 0.01$); B = significantly different from VAD adjusted for CRP-alone ($P < 0.01$).

In conclusion, the appropriate assessment of population vitamin A status is of global interest, not only because VAD remains a public health problem in several low-income countries, but also because of the need to estimate the impact of available VAD control programs. Our findings highlight an important criteria which can be incorporated into current models for characterizing IIH. It is important that the APPs for characterizing inflammation in similar settings be judged on the basis of their biological associations with retinol. We propose that models for characterizing inflammation take into consideration the variance in retinol explained by specific APPs and the consistency in adjusted VAD estimates over varying intensities of infection or inflammation. Our findings also highlight areas for additional research. There is a need to demonstrate the appropriateness of using CRP and AGP, either alone or in combination, for quantifying IIH in other settings. Furthermore, considering that vitamin A modulates the inflammatory response to infections,³⁷ including malaria,³⁸ there is a need for research into models that take into consideration the potential influence of baseline vitamin A status.

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