The Acute Phase Response Affected Traditional Measures of Micronutrient Status in Rural Zambian Children during a Randomized, Controlled Feeding Trial1,2

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Abstract

The acute phase response (APR) to infection can alter blood-based indicators of micronutrient status. Data from a 3-mo randomized, controlled feeding trial in rural Zambian children (n = 181, aged 3–5 y) were used to determine the impact of the APR on indicators of vitamin A and iron status using baseline and final blood samples. Concentrations of acute phase proteins were categorized as raised C-reactive protein (CRP; >5 and >10 mg/L) only, both raised CRP and α1-acid glycoprotein (AGP; >1.2 g/L), raised AGP only, and neither CRP nor AGP raised to identify the respective stages of infection: incubation, early convalescence, convalescence, and healthy state. Data were insufficient to examine the incubation stage of infection. A CRP concentration of >5 mg/L was an effective elevation cutoff point in this population to show impact on micronutrient markers. Time did not affect hemoglobin, serum ferritin, or serum retinol concentrations (P > 0.05). During early convalescence, hemoglobin decreased (14–16%; P ≤ 0.05), serum ferritin increased (279–356%; P ≤ 0.05), and serum retinol decreased (20–30%; P ≤ 0.05). Serum retinol concentrations did not change during convalescence; however, hemoglobin remained depressed (4–9%) and serum ferritin was elevated (67–132%) (both P ≤ 0.05). Modified relative dose response values were unaffected by the APR (P > 0.05) but increased between time points (16%; P ≤ 0.05), indicating a decrease in liver vitamin A reserves on the background of a semiannual vitamin A supplementation program. The observed prevalence of anemia and vitamin A deficiency assessed by serum retinol concentration was higher during the APR (P ≤ 0.05). It is important to consider the impact of infection on dietary interventions and to adjust for acute phase proteins when assessing iron status or vitamin A status by serum retinol concentration alone in children. J. Nutr. 144: 972–978, 2014.

Introduction

In public health assessments, micronutrient status is commonly determined using concentrations in blood as a surrogate for total body status. Blood concentrations of micronutrients can be altered by the acute phase response (APR) to infection (1,2). Whether this change is reflective of a redistribution of the micronutrient within the body, a negative shift in total stores, or a combination of both factors is a complex issue. Micronutrient demands tend to be increased during infection, and deficiency can increase susceptibility to, and be exacerbated by, chronic or recurrent episodes of febrile illness (3–6). However, substantial changes in micronutrient status do not likely occur as early as decreases in blood concentrations suggest during the APR. Additionally, blood concentrations are typically restored when the infection resolves (7–10), supporting measurements taken during the APR as inadequate reflections of micronutrient status. Therefore, it is paramount to consider the impact of the APR on blood-based micronutrient status indicators in populations with a high or unknown prevalence of febrile infection.

Acknowledgments

1 Supported by Global Health funds at the University of Wisconsin-Madison during manuscript preparation and HarvestPlus contract number 8217 for the fieldwork. HarvestPlus is a global alliance of agriculture and nutrition research institutions working to increase the micronutrient density of staple food crops through biofortification. The views expressed in this manuscript do not necessarily reflect those of HarvestPlus. This work was presented at the ASN Scientific Sessions at Experimental Biology 2014.


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Abbreviations used: AGP, α1-acid glycoprotein; APR, acute phase response; C–A−, neither CRP nor AGP raised; C–A+, AGP raised only; C+A−, CRP raised only; C+A+, both CRP and AGP raised; CRP, C-reactive protein; MRDR, modified relative dose response.
The APR is a systemic reaction to disruptions in the body’s homeostasis that is characterized by inflammatory mechanisms aimed at mitigating infection or trauma (11). Positive acute phase proteins that increase in the blood during infection can be used to assess the stage and severity of the response; C-reactive protein (CRP) and α1-acid glycoprotein (AGP) are commonly measured in nutrition interventions. CRP concentrations rise within 6 to 8 h of infection, reach maximal 10- to 1000-fold increases in concentration around 48 h, and normalize rapidly as infection resolves (12,13). AGP concentrations become elevated 24 to 48 h after initial infection with a maximum 4-fold increase in concentration around 5 to 6 d and can remain raised weeks after the infection convalesces (11,14). Both of these acute phase proteins are also raised during low-grade chronic inflammation, such as heart disease (15,16). Using these proteins, it is possible to identify different stages of infection including incubation (CRP only raised), early convalescence (both CRP and AGP raised), convalescence (AGP only raised), and to return to a healthy state (neither CRP nor AGP raised) (17).

We investigated the impact of the APR on blood-based indicators of vitamin A and iron status using data from baseline and final assessments of a 70-d maize feeding trial in rural Zambian children. Treatment arms included high provitamin A–biofortified (orange) and white maize. The effect of the APR was estimated for each micronutrient indicator investigated, and the consequence of infection on deficiency prevalence was determined. Prevalence of CRP elevation cutoffs of 5 and 10 mg/L are commonly reported in the literature (15,17–23); therefore, both values were examined. For AGP, 1.2 g/L was used as the raised cutoff point (21,24).

**Participants and Methods**

**Study design and location.** The feeding protocol used in this trial was previously published (25,26) and is summarized herein. All procedures involving human participants were approved by the Tropical Diseases Research Center Ethics Review Committee in Zambia and by the Health Sciences Human Subjects Institutional Review Board at the University of Wisconsin-Madison. Children 3–5 y of age (n = 181 at final enrollment) were recruited to participate in a randomized, controlled feeding trial with traditional white and high provitamin A carotenoid–biofortified (orange) maize. The mean participant age was 4.5 ± 0.9 y. Written informed consent was obtained from guardians of all participating children. Participants were screened and excluded from the study for severe anemia (hemoglobin <70 g/L) and malnutrition (≤-3 SD weight-for-age or height-for-age Z-scores) (27). The feeding trial was carried out in 6 villages in the Eastern Province of Zambia from March to June of 2010.

After being randomly assigned to the treatment groups, participating children were fed a standardized menu including breakfast, lunch, and snack 6 dwk for a total of 70 d of feeding, excluding Sundays. The only difference between the treatment groups was the type of maize used in the standardized menu. The orange maize contained 3.0 to 6.3 μg of theoretical vitamin A/g during the 70-d study (25). Overall, dietary intakes of macro- and micronutrients were similar between the white and orange maize groups (26). Data on the adaptation of children to orange maize (25) and on the impact of malaria on dietary intake are published (26).

Blood samples were collected at baseline and final time points. Zambia has an active, high-dose vitamin A supplementation program for children <5 y of age as recommended by the WHO (28). Therefore, the entire trial occurred after a high-dose supplement was consumed and finished a few days before administration of the next semiannual supplement. Children were only enrolled if they had consumed a supplement.

**Modified relative dose response test and serum retinol concentrations.** For the modified relative dose response (MRDR) test, a single oral dose of 5.3-μmol 3,4-didehydroretinyl acetate in corn oil was administered via positive displacement pipet to participants followed by consumption of peanut butter and bread to ensure adequate fat for efficient dose absorption. Blood samples were drawn 4 to 6 h after dosing using 5-mL Vacutainer tubes (Becton Dickinson). Samples were placed on ice until serum could be separated by centrifugation; serum was transported in nitrogen vapor in a dry shipper and then stored at ~70°C until analysis. Samples were analyzed simultaneously for MRDR value and serum retinol concentration using a standardized method (29). Serum (200 μL) was treated with 250-μL ethanol and extracted twice with hexane; retinyl acetate served as the internal standard. Pooled hexanes were dried under nitrogen and reconstituted in 40-μL 75:25 methanol/dichloroethane for injection (35 μL) onto an HPLC system equipped with a Waters Resolve C18 column (3.9 × 150 mm, 5 μm). The mobile phase of 89:11 methanol/water (0.73 g/L triethylamine) was run at a flow rate of 1 mL/min. The definition for vitamin A deficiency using the MRDR test was considered a serum molar ratio of 3.4-didehydroretinol: retinol ≥0.060 (29,30).

**Hemoglobin, serum ferritin, and markers of infection.** At the time of blood draw, hemoglobin concentrations of whole blood were determined using a portable hemoglobinometer (Hemocue). Serum ferritin concentrations were measured using a commercially available ELISA kit (Ratco). The absorbance of all samples was verified at 510 and 630 nm on a Tecan Sunrise ELISA reader (Model A 5080; Tecan Group). CRP and AGP standards were used to create calibration curves for the conversion of precipitin complex diameters to serum concentrations. Methods for the determination of malaria parasitemia are published (26).

**Statistical analysis.** Values are means ± SDs. Data were analyzed using SAS software (SAS Institute). Serum ferritin concentrations were transformed via logarithm before statistical analysis. Baseline and final anthropometric and biochemical data were compared within and between white and orange maize groups using paired and independent t tests, respectively. Correlations between markers of micronutrient status concentrations and acute phase proteins were assessed with Pearson’s correlation. Separately investigating elevation cutoffs for CRP of >5 and >10 mg/L, differences in micronutrient status among participants with neither CRP nor AGP raised (C–A–), CRP raised only (C+A–), both CRP and AGP raised (C+A+), and AGP raised only (C–A+) were analyzed by time using 2-factor ANOVA tests. Differences between groups were determined using least-square differences at α = 0.05. The effect of gender on hemoglobin and serum ferritin values was investigated and excluded because it was not significant (both P > 0.05). The C+A– group was excluded because the sample size (n = 4) did not yield enough observations to justify analysis. Therefore, the total population included in the overall and 2-factor ANOVA tests was decreased by a total of 5 and 1 observations, respectively, for CRP elevation cutoffs of >5 and >10 mg/L, respectively. Differences in the prevalence of micronutrient deficiency were determined within a time point by directly comparing groups with elevated acute phase proteins to the group without raised acute phase proteins using Fisher’s exact test. Only participants with a complete data set for markers of infection (n = 178) at initial and final time points were included in ANOVA and Fisher’s exact tests.

Sample size calculations were based upon a projected change in the most sensitive marker of vitamin A status used, the MRDR test. A sample size of 80 was needed to measure a difference of 0.02 in the MRDR value, assuming a SD of 0.04 in each group at a 5% significance level and 90% power. This sample size was used in a trial in South African children for a sweet potato intervention, and the MRDR showed an intervention effect (30). To account for dropouts during the trial, we aimed to recruit 100 children in each group.
Results

Participant data. Overall, 14 children dropped out of the study (7%; Fig. 1). Primary reasons for discontinuing study participation included noncompliance with study requirements and severe illness. Data were pooled by time point for white and orange maize groups because no differences were observed between groups with the exception of initial serum ferritin, which was higher in the orange maize group (198 ± 232 μg/L) than the white maize group (128 ± 131 μg/L) (Table 1). No change was seen in the weight-for-height Z-score; however, weight and height increased by 6.1% and 2.3%, respectively, during the study. Malaria prevalence decreased by 8.4%, likely because of the transition out of malaria season that occurred during the trial (26). Serum CRP concentration did not change; the prevalence of elevated CRP decreased by 2.8% and 0.5% using the >5 and >10 mg/L cutoffs, respectively. Serum AGP concentration decreased by 17.6%, which corresponded to a 27.0% decrease in the prevalence of elevated AGP at the final assessment. Of participants with malaria (n = 37 at baseline; n = 22 at final), 78.4% and 59.1% had raised acute phase proteins using a CRP cutoff of 5 mg/L, and 78.4% and 54.6% using a CRP cutoff of 10 mg/L at initial and final time points, respectively.

Comparison of micronutrient status concentrations or values across infection stage. Similar correlations between markers of interest were found at initial and final time points (Table 2). Both hemoglobin and serum retinol concentrations correlated negatively with CRP and AGP concentrations. A positive correlation was found between serum ferritin and acute phase protein concentration. No correlational relation was found between MRDR value and acute phase protein concentration. CRP and AGP were positively correlated. Hemoglobin and serum ferritin shared a negative association, suggesting a shift in iron distribution during infection.

The overall test revealed differences among infection groups in all investigated micronutrient indicators (all \( P < 0.05 \)) (Table 3). The 2-factor ANOVA analysis revealed a significant effect of infection stage, but not a time effect or an interaction between infection stage and time for hemoglobin, serum ferritin, and serum retinol concentrations. Similar patterns of significance were also discovered using both CRP elevation cutoffs of >5 and >10 mg/L for all micronutrient indicators investigated. Compared with the C−A− group, hemoglobin measures were suppressed in both the C+A+ and C−A+ groups. Serum ferritin concentrations were markedly higher in groups with elevated acute phase proteins; the C+A+ group was 349% and 353% elevated, and the C−A+ group was 67% and 132% elevated at initial and final time points, respectively, when compared with the C−A− group for the CRP cutoff of 5 mg/L. Serum retinol concentration was lower in the C+A+ group, but the C−A+ group did not differ from the C−A− group. For the MRDR value, a significant effect of time was observed but no effect of infection stage or an interaction. MRDR values were higher at the final assessment point when compared with the initial assessment, which indicates a decrease in vitamin A liver reserves over time on the background of semiannual high-dose vitamin A supplementation. The mean MRDR value indicated
adequate concentrations of liver vitamin A at both initial and final time points (<0.060; P < 0.0001), but the prevalence of children above this cutoff increased at the end of the intervention (Table 4).

**Comparison of deficiency prevalence across infection stage.** Similar patterns of significance were discovered for both CRP cutoffs of 5 and 10 mg/L. Anemia prevalence at the initial time point was elevated in the C+A+ group. At the final measurement, the C+A+ and C−A+ groups were found to have inflated estimations of anemia. The prevalence of iron deficiency anemia was low, and no difference was found among groups

**TABLE 2** Correlations between markers of micronutrient status and acute-phase proteins in Zambian children at initial and final time points of a 70-d feeding trial

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Initial</th>
<th>Final</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>14.8 ± 1.9</td>
<td>15.7 ± 2.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Height, cm</td>
<td>98.4 ± 6.9</td>
<td>100.7 ± 6.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Weight-for-height Z-score2</td>
<td>0.008 ± 0.88</td>
<td>0.010 ± 0.87</td>
<td>0.98</td>
</tr>
</tbody>
</table>

**Markers of infection**

- **Malaria prevalence**, %: 20.8, 12.4, —
- **Parasite load >0**: 17.4, 11.2, —
- **Parasite load >1000**: 3.4, 1.2, —
- **CRP**, mg/L: 2.88 ± 3.47, 3.04 ± 4.30, 0.67
- **Elevated >5, %**: 15.7, 12.9, —
- **Elevated >10, %**: 6.7, 6.2, —
- **AGP**, g/L: 1.42 ± 0.53, 1.17 ± 0.50, <0.0001
- **Elevated >12, %**: 85.2, 38.2, —

1 Values are means ± SDs or percentages; n = 181. The data for orange and white maize groups were combined because no differences were observed (all P > 0.05), with the exception of initial serum ferritin, which was higher in the orange maize group (198 ± 232 μg/L) than the white group (128 ± 131 μg/L) (P = 0.015); Zambian children were aged 53.7 ± 10.2 mo at baseline. AGP, α−acid glycoprotein; CRP, C-reactive protein.

2 WHO Child Growth Standards, ages 2−6 y (31).

3 Overall malaria prevalence did not differ by treatment group or study week (P > 0.05); a decrease in total malaria prevalence was found when comparing the extremes (P < 0.041) (26).

4 Parasite counts per 200 white blood cells.

5 Raised serum concentrations of acute-phase proteins were defined as CRP >5 or >10 mg/L and AGP >1.2 g/L (11,18-24).

**Discussion**

The APR alters blood-based micronutrient status indicators, making it crucial to consider the impact of infection on the outcomes of nutrition interventions. This phenomenon was described in previous studies; however, a limited body of work focuses on the quantification of infection’s impact on micronutrient status indicators, especially in areas with endemic malaria. The current study adds to the pool of data available for the development of standardized methods for the adjustment of micronutrient status indicators during the APR. In this study, the effect of the APR on hemoglobin, serum ferritin, serum retinol, and MRDR value was quantified in rural Zambian children at baseline and final time points of a 70-d feeding trial. Data were insufficient to investigate the impact of the incubation stage of infection (C−A−) because most children had elevated AGP living in this rural environment. The observed prevalence of anemia and vitamin A deficiency assessed by serum retinol concentration was significantly higher during the APR. Iron deficiency anemia prevalence was very low (1.0−4.1%), likely due in part to the marked inflation of 67 to 353% in serum ferritin values during infection. The MRDR value was unaffected by the APR, which is in agreement with one former report of this observation (21). The APR should be considered when measuring micronutrient status indicators that are reactive to it.

Blood-based micronutrient indicators are affected during the APR through the hepatic suppression of transport proteins (e.g., retinol-binding protein and transthyretin) and an increase in serum ferritin, itself a positive acute phase protein that assists iron sequestration (2,3,36). The magnitude of the APR varies by time stage and severity of infection. As reported in this study and by others, serum retinol and hemoglobin concentrations are negatively, and serum ferritin is positively, correlated with CRP and AGP concentration in children (10,18,24,37,38). Additionally, Duncan et al. (39) recently demonstrated that for serum retinol, and several other micronutrients assessed in the blood, the extent of alteration corresponded to the magnitude of the APR when assessed by CRP concentration in adults.

Serum retinol and ferritin concentrations typically return to preinfection values as the APR resolves (7−10) but are affected differently by the time stage of infection. Louw et al. (8) found changes in serum retinol concentrations to correspond to CRP concentration in patients who underwent orthopedic surgery. Serum ferritin concentrations remain elevated after CRP values normalized and AGP was still raised (9,21,40). Similar patterns of alteration during the APR were found for serum retinol and ferritin concentrations in the present study. Hemoglobin was suppressed in the current study and was also discovered to be so in rural British and Zanzibari children during the APR (38,41). Additionally, hemoglobin was previously, and in this work, found to be inversely related to serum ferritin (38), supporting its role as a reactive iron status indicator. However, Wieringa et al. (21) reported no effect of the APR on hemoglobin, and Das...
et al. (42) found no correlation between hemoglobin and serum ferritin. Because the impact of the APR on hemoglobin is unclear, more work is needed to elucidate this relation.

CRP cutoffs of 5 or 10 mg/L are typically used to detect clinically relevant infections for a wide variety of micronutrients and populations (15,17–23); however, cutoffs relevant to shifts in micronutrient status indicators may be nutrient and age specific. Duncan et al. (39) found CRP cutoffs between 5 and 20 mg/L to be necessary to detect changes in blood concentration for various vitamins and minerals in adults, whereas Abraham et al. (43) found significant changes in blood nutrients, including serum ferritin and retinol, occurring at CRP concentrations of merely 0.26 mg/L in infants. CRP concentrations increase with age and tend to be higher in women than men (44). This increase

| Table 4 | Initial and final prevalence of micronutrient deficiencies by infection state as determined by acute phase proteins in Zambian children during a 70-d randomized, controlled trial |

<table>
<thead>
<tr>
<th>Markers of infection</th>
<th>CRP &gt;5 mg/L</th>
<th>CRP &gt;10 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>g/L</td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>178</td>
<td>102 ± 14</td>
</tr>
<tr>
<td>C–A–</td>
<td>61</td>
<td>106 ± 13</td>
</tr>
<tr>
<td>C+A–</td>
<td>2</td>
<td>95</td>
</tr>
<tr>
<td>C+A+</td>
<td>27</td>
<td>91 ± 14</td>
</tr>
<tr>
<td>C–A+</td>
<td>89</td>
<td>102 ± 13</td>
</tr>
</tbody>
</table>

| Final                |             |              |              |              |             |             |              |              |              |             |
| Total                | 178         | 105 ± 15     | 155 ± 277     | 0.96 ± 0.34   | 0.050 ± 0.028 | 178         | 105 ± 15     | 157 ± 277     | 0.96 ± 0.34   | 0.050 ± 0.028 |
| C–A–                 | 106         | 110 ± 12     | 86.7 ± 12.2   | 1.02 ± 0.31   | 0.045 ± 0.15ab | 109         | 110 ± 12     | 92.6 ± 139    | 1.02 ± 0.32   | 0.045 ± 0.15ab |
| C+A–                 | 4           | 112 ± 16     | 266 ± 309     | 0.73 ± 0.15   | 0.060 ± 0.014a | 1           | 101         | 101           | 0.72          | 0.071        |
| C+A+                 | 19          | 92 ± 18      | 392 ± 463     | 0.71 ± 0.32   | 0.065 ± 0.018a | 10          | 96 ± 16      | 351 ± 327     | 0.68 ± 0.29   | 0.057 ± 0.013a |
| C–A+                 | 49          | 100 ± 14     | 201 ± 365     | 0.95 ± 0.36   | 0.068 ± 0.046a | 58          | 98 ± 16      | 238 ± 405     | 0.92 ± 0.37ab | 0.057 ± 0.043a |

| P values             |             |              |              |              |             |             |              |              |              |             |
| Overall              |             | <0.0001      | <0.0001      | 0.0008       | 0.016       | <0.0001     | <0.0001      | 0.0052       | 0.012        |
| Infection effect     |             | <0.0001      | <0.0001      | 0.0001       | 0.39        | <0.0001     | <0.0001      | <0.0001      | 0.33         |
| Time effect          | 0.61         | 0.49         | 0.37         | <0.0001      | 0.20        | 0.23        | 0.62         | 0.0057       |
| Interaction          | 0.22         | 0.60         | 0.69         | 0.17         | 0.078       | 0.47        | 0.90         | 0.17         |

1 Values for micronutrient status are means ± SDs. Groups with n ≤ 4 were excluded from the ANOVA. Serum ferritin was analyzed using logarithmic transformation. Letters within a column, excluding values for total population, indicate significant differences determined by least square difference. AGP, α2-acid glycoprotein; C–A–, neither CRP nor AGP raised; C–A+, AGP raised only; C+A–, CRP raised only; C+A+, both CRP and AGP raised; CRP, C-reactive protein; MRDR, modified relative dose response.

2 Raised serum concentrations of acute phase proteins were defined as CRP >5 mg/L and AGP >1.2 g/L (15,18–24).
is in part related to chronic inflammatory conditions (15,16), suggesting that a higher CRP cutoff may be necessary to assess acute infections in adults. In the current study, no differences were discovered in sensitivity between 5- and 10-mg/L CRP cutoffs, supporting the usefulness of the 5-mg/L cutoff for vitamin A and iron status assessments in children when AGP is also elevated. More research aimed at investigating CRP cutoffs of ≤5 mg/L on micronutrient status indicators in children is needed and should include a variety of kits and tests available. Additionally, AGP identified more cases of infection than CRP (74.7–91.3% vs. 8.7–25.3%) in this study because AGP typically remains elevated for a longer period of time, and children have recurrent infections in many developing countries. The decrease in the prevalence of elevated AGP concentration between baseline and final time points is likely reflective of the decrease in malaria prevalence caused by seasonal effects (26). Assessment of both early- and late-stage acute phase proteins is essential to gain a true understanding of infection within a population when using traditional measures of status.

The MRDR test relies on the principle that retinol binding protein accumulates when vitamin A liver reserves are low and is measured by the ratio of 3,4-didehydroretinol:retinol in serum (29,30,32). The present finding that the MRDR value is unaffected by the APR is supported by Wieringa et al. (21), who similarly reported no change through APR time stages measured by CRP and AGP in Indonesian children. The lack of difference in MRDR value across infection stage implies that retinol binding protein continues to accumulate during deficiency despite infection stage and is present in the liver to bind with 3,4-didehydroretinol during a short-term challenge test. Furthermore, this finding suggests that 3,4-didehydroretinol release is somewhat suppressed proportionally to retinol, such that the ratio between the two remains the same.

Serum retinol concentration is a widely used indicator for vitamin A status and is recommended for population assessment by the WHO (33). However, serum retinol provides an inferior vitamin A status and is recommended for population assessment that the ratio between the two remains the same.

In conclusion, this study’s findings demonstrate that the APR significantly alters blood-based indicators of population-based iron and vitamin A status indicators in children, resulting in over- and possibly underestimations of nutrient deficiency prevalence. Early- and late-stage acute phase proteins, such as CRP and AGP, can be effectively employed to assess infection stages; a CRP elevation cutoff of 5 mg/L was sufficient in this population to affect serum-based indicators. Incorporating blood measurements of acute phase proteins will improve micronutrient status assessment.

Acknowledgments
The authors thank Peter Crump (University of Wisconsin-Madison College of Agricultural and Life Sciences Statistical Consulting Service) for statistical analysis consultation; Kevin Pixley and Natalia Palacios Rojas for breeding, producing, and sending sufficient high provitamin A seed for grain production in Zambia; Natalia Palacios Rojas for assisting with fieldwork; Bhola Verma for securing land for grain production; Raphael Mutale and Elia Simpungwe for coordination of maize grain production in Zambia; and Bryan Gannon for proofreading the manuscript. K.A.B. analyzed data and wrote the manuscript. K.A.B., S.A., E.N., and R.S. conducted the fieldwork; J.C. coordinated the blood sample collection and storage as well as analysis of blood samples for acute phase proteins and micronutrient status indicators; R.S. standardized the Tropical Diseases Research Center laboratory; and C.M. oversaw the staff at the National Food and Nutrition Commission and worked with district officers for conduct of the trial. As co-principal investigator, E.K. (deceased 17 May 2010) was essential in setting up this study in Zambia. S.A.T. designed the research, provided oversight at the field stations during the trial, provided input for the statistical analysis, and revised the manuscript. All authors read and approved the final manuscript.

Literature Cited

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