High provitamin A carotenoid serum concentrations, elevated retinyl esters, and saturated retinol-binding protein in Zambian preschool children are consistent with the presence of high liver vitamin A stores[1]

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ABSTRACT

Background: Biomarkers of micronutrient status are needed to best define deficiencies and excesses of essential nutrients.

Objective: We evaluated several supporting biomarkers of vitamin A status in Zambian children to determine whether any of the biomarkers were consistent with high liver retinol stores determined by using retinol isotope dilution (RID).

Design: A randomized, placebo-controlled, biofortified maize efficacy trial was conducted in 140 rural Zambian children from 4 villages. A series of biomarkers were investigated to better define the vitamin A status of these children. In addition to the assessment of total-body retinol stores (TBSs) by using RID, tests included analyses of serum carotenoids, retinyl esters, and pyridoxal-5′-phosphate (PLP) by using high-pressure liquid chromatography, retinol-binding protein by using ELISA, and alanine aminotransferase (ALT) activity by using a colorimetric assay.

Results: Children (n = 133) were analyzed quantitatively for TBSs by using RID. TBSs, retinyl esters, some carotenoids, and PLP differed by village site. Serum carotenoids were elevated above most nonintervened reference values for children. α-Carotene, β-carotene, and lutein values were >95th percentile from children in the US NHANES III, and 13% of children had hypercarotenemia (defined as total carotenoid concentration >3.7 μmol/L). Although only 2% of children had serum retinyl esters >10% of total retinol plus retinyl esters, 16% of children had >5% as esters, which was consistent with high liver retinol stores. Ratios of serum retinol to retinol-binding protein did not deviate from 1.0, which indicated full saturation. ALT activity was low, which was likely due to underlying vitamin B-6 deficiency, which was confirmed by very low serum PLP concentrations.

Conclusions: The finding of hypervitaminosis A in Zambian children was supported by high circulating concentrations of carotenoids and mildly elevated serum retinyl esters. ALT-activity assays may be compromised with co-existing vitamin B-6 deficiency. Nutrition education to improve intakes of whole grains and animal-source foods may enhance vitamin B-6 status in Zambians. This trial was registered at clinicaltrials.gov as NCT01814891. *Am J Clin Nutr* 2015;102:497–504.

INTRODUCTION

Vitamin A (VA)[7] status assessment of humans is challenging because liver VA concentrations are considered the gold standard of VA status (1) but are difficult to assess. Serum retinol (SR) concentrations are homeostatically controlled over a wide range of liver reserves (2) and are decreased during the acute-phase response (3, 4). Retinol isotope dilution (RID) is currently considered the most-sensitive indirect biomarker of VA status (1, 2) and can be used to determine the response to interventions (5); however, a quantitative estimation of total-body retinol stores (TBSs) or total liver reserves requires the numerical estimation of dose absorption, partitioning in organs, and catabolism and excretion (6). Researchers have used different methodologies and assumptions when calculating VA status by using RID (6). Because of surprising findings that indicated adequate through hypervitaminotic VA status of Zambian preschoolers (7), these assumptions have been challenged (8). Therefore, other biomarkers of VA status were investigated to qualify the findings of high liver reserves in this group of children. Additional evidence of actual VA status of a group could be evaluated with valid dietary and health biomarkers that can be applied at the population level to support quantitative measurements.

The distribution and concentration of carotenoids varies widely in fruit and vegetables (9). Some carotenoids are precursors of VA that can be bioconverted by humans and other animals; the most common of these carotenoids in the human diet are α-carotene, β-carotene, and β-cryptoxanthin. Other carotenoids in human circulation cannot be converted into VA but serve other physiologic purposes (e.g., lutein in eye health) (10), and serum concentrations can be used to verify specific vegetables or fruit in the diet. Plant sources of provitamin A carotenoids are a major source of VA and have been estimated at providing a substantial percentage ($\sim$68%) of total worldwide VA (11). Serum carotenoid concentrations are a function of a number of factors, the most pertinent being dietary intake (5, 12–15). Other factors associated with serum carotenoids are age, BMI, and the genetic variation in enzymes related to carotenoid absorption, transport, cleavage, and degradation (16). Provitamin A carotenoid bioefficacy is inversely related to VA status (5) and the VA content of the diet (17), likely mediated by the VA-induced negative feedback of carotenoid transporter scavenger receptor-B1 and cleavage enzyme β-carotene 15,15'-oxygenase (BCO1) by transcription factor intestine-specific homeobox (18, 19). Plasma carotenoids and TBSs are increased in response to consumption of high-carotenoid diets (5, 12, 14). Skin and serum carotenoids reflected both a low-carotenoid regimen followed by a high-carotenoid regimen (20).

Serum retinyl esters are used as a biomarker of high VA stores although the limitations of this assessment in children are not clear because the cutoff of 10% of the total as retinyl esters was chosen on the basis of adults with unknown TBSs. The retinyl ester concentration was first suggested as a biomarker on the basis of 3 patients with chronic intakes of pharmaceutical doses that caused hypervitaminosis A (21). The presentation of retinyl esters in lipoproteins to cells instead of retinol on retinol-binding protein (RBP) is hypothesized to cause VA toxicity (21, 22). Hypervitaminosis A leads to liver fibrosis and elevated liver enzymes in plasma. Additional assessments in Zambian children were performed to gain more insight into the degree of hypervitaminosis A present (7).

METHODS

Subjects

All field procedures involving children were approved by the Ethics Review Committee of the Tropical Diseases Research Centre (TDRC) in Zambia and the Health Sciences Human Subjects Institutional Review Board of the University of Wisconsin-Madison. This trial was registered at clinicaltrials.gov as NCT01814891; outcomes related to the intervention have been reported (7), and biomarkers reported herein are before the intervention except for deworming, which was performed 1 wk before the first blood sample. Written informed consent was obtained from parents or caregivers. The trial was conducted in 2012 in the Nyimba District of the Eastern Province of Zambia in preschool children ($n = 143$ at initial enrollment) because of a high prevalence of low SR concentrations in a previous survey (23). The following 4 sites were chosen: 2 sites adjacent to the main paved roadway (coded as sites A and B) and 2 sites $\sim$8 km off the paved road (coded as sites C and D).

Inclusion criteria were as follows: 5–7-y-old children living in the study area who were considered relatively healthy (no clinical infection or fever, weight-for-age and weight-for-height z scores greater than -3, and hemoglobin concentration >70 g/L at recruitment), who had received antihelminthic treatment the week before recruitment, and had not received a high-dose VA supplement in the past 6 mo. Blood collection (7 mL) was performed by the TDRC, followed by centrifugation at the local clinic. Malaria parasites were counted on thick blood smears prepared in the field as described (24). Serum was transferred into 2 tubes, transported in nitrogen gas to the TDRC, shipped on dry ice, and stored at $-80°C$ until analysis at the University of Wisconsin-Madison for all VA biomarkers or the University of Florida for concentrations of pyridoxal-5'-phosphate (PLP), which is the physiologically active form of vitamin B-6.

TBSs and liver concentrations of VA

TBSs and liver concentrations of VA were determined by using ^{13}C-RID and applying the mass balance equation with the following assumptions: 90% dose absorption, fractional catabolic rate of 0.5%/d during the mixing period, equal serum and liver ^{13}C-enrichment, and 80% of TBSs in the liver (7). A decrease to 80% absorption was made for children with elevated CRP at the time of dosing. After a baseline blood draw, 1 μmol ^{13}C$_2$-retinyl acetate dissolved in soybean oil was delivered directly to each child by using a positive-displacement pipette and immediately followed by a high-fat–containing snack to facilitate absorption. After a 14-d mixing period during which subjects consumed a controlled diet with limited VA (25), a second blood sample was taken and the ^{13}C:total C of SR at both blood draws was determined by using gas chromatography–combustion isotope ratio mass spectrometry to estimate TBSs and liver retinol concentrations (7).

Carotenoid and retinyl ester extraction and analysis

Samples were extracted for carotenoids and retinyl esters by using a modified published procedure (26). To 1 mL (or all available) serum, ethanol (1.5 × volume) with 0.1% butylated hydroxytoluene as an antioxidant and 100 μL C23 β-apo-carotenol as an internal standard were added. Samples were extracted 3 times with 1.5-mL hexanes. Pooled hexane layers were dried under nitrogen and reconstituted in 100 μL 50:50 (volume:volume) methanol:dichloroethane. To have high sensitivity to detect some of the minor retinyl esters and to ensure good separation of carotenoids, aliquots of the same extract were run on 2 separate HPLC systems.

For carotenoid analysis, 25 μL extract was injected onto a Waters HPLC system (Waters) comprised of a C18 Resolve (5-μm, 3.9 × 300-mm) analytic column (Waters) equipped with a guard column, 2707 autosampler, 1525 binary pump, and 2998 photodiode array detector. Samples were eluted at 2 mL/min by using 95:5 (volume:volume) acetonitrile:water (solvent A) and 85:10:5 (volume:volume:volume) acetonitrile:methanol:dichloroethane (solvent B) both with 10 mmol ammonium acetate/L as a modifier by using the following gradient method: 3 min at 100% A, followed by a 7-min linear gradient to 100% B, a 15-min hold at 100% B, 1-min linear gradient back to 100% A, and a 5-min hold

at 100% A for re-equilibration. Chromatograms were evaluated at 450 nm by using authentic HPLC-purified standards.

For the analysis of retinyl esters, 50 μL serum extract was injected onto a Waters C18 Resolve (5-μm, 3.9 × 300-mm) column equipped with guard column. A Waters Delta 600 binary pump and controller (Waters), 2487 Dual-Wavelength Absorbance Detector (Waters), and a CR7A Chromatopac data processor (Shimadzu) comprised the HPLC system. Chromatograms were generated at 325 nm to quantify retinol and retinyl esters, which were confirmed by retinyl ester standards isolated and purified from pig liver. The mobile phase was 1.5 mL 85:15 (volume: volume) acetonitrile:water/min with 10 mmol/L ammonium acetate (solvent A) as an initial condition followed by a 10-min linear gradient to 100% 80:20 (volume:volume) acetonitrile: dichloroethane (solvent B). Solvent B was held for 12 min followed by a 2-min linear reverse gradient to 100% A and an 8-min hold at 100% A.

Other assays

Serum C-reactive protein (CRP) (Cayman Chemical Co.), α_1-acid glycoprotein (AGP) (Abcam), and RBP (Arbor Assays), all of which are acute-phase proteins, were assayed by using enzyme immunoassay kits. Alanine aminotransferase (ALT) activity was assayed by using a colorimetric assay kit as recommended by the manufacturer (Sigma-Aldrich) as part of a strategy to gauge if any hepatocellular damage had occurred from excessive storage of retinyl esters in the liver. Once it was shown that ALT activity was actually lower than normal, an inquiry into vitamin B-6 status, which is a cofactor for ALT function, was added to the protocol. With collaboration at the University of Florida, PLP was determined by using HPLC followed by fluorescence detection (27).

Statistical analysis

Data are reported as medians (first and third quartile values, which are equivalent to 25th and 75th percentiles, respectively) to control nonnormality in some outcome measures or means ± SDs for in-text summaries. Data were analyzed by using the General Linear Model procedure in the Statistical Analysis System (version 9.4; SAS Institute). The normality of residuals was assessed by using the Shapiro-Wilk test, and the homogeneity of variance was assessed by using Levene's test. For data that satisfied assumptions, outcomes of interest were evaluated by using 1-factor ANOVA, and differences in study sites were determined by using least-significant difference tests. For data that failed assumptions, a nonparametric analysis was carried out on ranked data. Proportions were compared by using χ^2 analysis. Significance was defined as $P \leq 0.05$.

RESULTS

Subject characteristics

Enrollment occurred at the end of May 2012. Children ($n = 143$) were recruited and consented, and 140 children met baseline inclusion criteria and had blood samples taken (7). Because of statistically significant effects of site on nutritional outcome measures, baseline anthropometric data are presented by site (**Table 1**). Of particular interest was that village B did not have any cases of asymptomatic malaria, which was related to higher hemoglobin concentrations than in other villages ($P < 0.0001$). Although enrollment occurred during the dry season when malaria transmission is low, children with active malaria would not have been enrolled because of exclusion criteria.

Markers of infection status were evaluated (7). Although elevated AGP was universal in villages, CRP differed by site and was lower in villages closer to the paved road ($P < 0.0001$) (**Table 2**).

Total liver retinol reserves

Calculated mean liver reserves for all subjects were 1.13 ± 0.41 μmol retinol/g liver, with 59% >1 μmol/g (7), which is the current cutoff for defining hypervitaminosis A (2). No reserves were <0.1 μmol/g, which is the deficiency cutoff (2). Village B had statistically significantly higher TBSs than those of villages located farther from the main road (i.e., villages C and D) (Table 2).

Serum carotenoid concentrations

After the primary outcome analysis (7), 123 samples had sufficient serum to quantify the carotenoid profile and retinyl esters. Carotenoids were not statistically significantly affected

TABLE 1
Baseline anthropometric and screening data for Zambian children ($n = 140$) by village[1]

	A ($n = 29$)	B ($n = 36$)	C ($n = 35$)	D ($n = 40$)	P
Age,[2] mo	72 (65, 80)[3]	68.5 (66.5,76)	69 (64, 75)	73 (66.5, 78.5)	0.34
Height, cm	107 (104, 113)	108 (105, 112)	107 (103, 110)	108 (103, 112)	0.57
Weight,[2] kg	16.4 (15.4, 18.7)	17.5 (15.6, 18.7)	16.8 (15.8, 18.2)	16.9 (16.0, 18.6)	0.76
Height-for-age z score	−1.2 (−2.1, −0.9)	−1.3 (−2.0, −0.7)	−1.6 (−2.2, −0.8)	−1.5 (−2.0, −1.1)	0.82
Weight-for-age z score	−1.4 (−1.9, −1.0)	−1.0 (−1.7, −0.5)	−1.3 (−1.8, −0.8)	−1.3 (−1.8, −0.8)	0.48
BMI-for-age z score	−0.6 (−0.9, −0.3)	−0.4 (−0.6, 0.1)	−0.4 (−0.8, 0.2)	−0.4 (−0.7, 0.0)	0.14
Hemoglobin,[2] g/L	118 (109, 123)[b]	125 (116, 128)[a]	117 (108, 123)[b]	112 (102, 120)[b]	<0.0001
Positive malaria blood smear, %	17.2	0	17.2	12.5	<0.0001

[1]Villages A and B were closest to the paved road, and villages C and D were 8 km from the road. Groups with uncommon superscript letters were different: a > b. P values are for testing the null hypothesis that each variable was equal in groups by using an ANOVA or chi-square test.
[2]Nonnormally distributed residuals; P value reflects a nonparametric analysis.
[3]Median; first and third quartile values in parentheses (all such values). First and third quartile values are also known as 25th and 75th percentiles, respectively.

TABLE 2
The following nutritional biomarkers were analyzed in Zambian preschool children[1]

	A	B	C	D	P
Total-body stores,[2] μmol	740 (483, 973) [27][a,b,3]	796 (671, 922) [34][a]	668 (512, 803) [34][b]	640 (510, 781) [38][b]	0.031
Estimated liver concentration,[2] μmol/g	1.05 (0.82, 1.54) [27]	1.17 (0.98, 1.49) [34]	1.06 (0.78, 1.28) [34]	1.01 (0.83, 1.11) [38]	0.068
Serum retinol,[2] μmol/L	0.95 (0.79, 1.16) [27]	1.00 (0.88, 1.19) [36]	0.90 (0.65, 1.05) [32]	0.96 (0.75, 1.12) [36]	0.24
Serum RBP, μmol//L	0.96 (0.79, 1.14) [19]	1.18 (0.85, 1.47) [18]	0.86 (0.61, 1.32) [18]	0.90 (0.67, 1.28) [23]	0.70
Retinol:RBP, molar ratio	0.96 (0.86, 1.10) [19]	1.02 (0.89, 1.20) [18]	0.95 (0.87, 1.09) [18]	1.04 (0.86, 1.21) [22]	0.45
Retinyl esters,[2] molar percentage of serum VA	3.0 (1.4, 3.7) [29][b]	4.0 (2.2, 5.2) [31][a]	1.5 (1.2, 2.7) [29][b]	2.6 (1.4, 3.6) [32][b]	0.0009
β-Carotene,[2] μmol/L	0.65 (0.50, 1.00) [29]	0.74 (0.51, 1.14) [31]	0.61 (0.40, 0.91) [27]	0.57 (0.29, 1.00) [32]	0.42
α-Carotene,[2] μmol/L	0.49 (0.35, 0.62) [29][b]	0.81 (0.47, 1.06) [31][a]	0.46 (0.29, 0.73) [30][b]	0.45 (0.26, 0.67) [32][b]	0.0016
β-Cryptoxanthin,[2] μmol/L	0.07 (0.05, 0.10) [27]	0.07 (0.03, 0.19) [22]	0.07 (0.05, 0.12) [24]	0.10 (0.07, 0.13) [28]	0.60
Lutein,[2] μmol/L	0.95 (0.67, 1.23) [29][a,b]	0.50 (0.39, 0.67) [31][c]	0.77 (0.41, 1.15) [30][b,c]	0.98 (0.81, 1.23) [33][a]	0.0003
Zeaxanthin,[4] μmol/L	0.04 (0.03, 0.06) [23]	0.02 (0.02, 0.03) [14]	0.04 (0.02, 0.06) [23]	0.04 (0.03, 0.06) [23]	0.07
Lycopene,[2,4] μmol/L	0.13 (0.09, 0.16) [17][c]	0.34 (0.26, 0.57) [22][a]	0.11 (0.10, 0.37) [9][b,c]	0.20 (0.12, 0.54) [14][a,b]	<0.0001
ALT activity,[2] U/L	3.3 (2.8, 4.1) [19]	3.1 (2.6, 3.5) [24]	2.8 (2.4, 3.6) [25]	2.7 (2.2, 4.1) [20]	0.48
PLP,[2] nmol/L	16.7 (11.9, 21.1) [26][a]	15.6 (11.2, 19.1) [22][a]	9.1 (7.1, 14.1) [21][b]	13.2 (8.5, 18.2) [21][a,b]	0.0044
Elevated CRP (>10 mg/L), % [n]	7.1 [28]	12.9 [31]	31.0 [29]	19.4 [36]	<0.0001
Elevated AGP (>1.2 g/L), % [n]	93.1 [29]	93.5 [31]	92.9 [28]	97.4 [38]	1.0

[1]Some biomarkers differed by site, which may have reflected local consumption of some foods. Villages A and B were closest to the paved road, and villages C and D were 8 km from the road. Carotenoid values were reported only for separately identifiable peaks. Groups with uncommon superscript letters were different: a > b > c. P values are for testing the null hypothesis that each variable was equal in treatment groups by using an ANOVA or chi-square test. AGP, $\alpha_1$acid glycoprotein; ALT, alanine aminotransferase; CRP, C-reactive protein; RBP, retinol binding protein; VA, vitamin A.

[2]Nonnormally distributed residuals; P value reflects a nonparametric analysis.

[3]Median; first and third quartile values in parentheses; n in brackets (all such values). First and third quartile values are also known as 25th and 75th percentiles, respectively.

[4]Values were not always quantifiable in samples that were <100 μL serum.

by age, sex, BMI, CRP, or AGP; however, 3 individual carotenoids (i.e., α-carotene, lutein, and lycopene; $P \leq 0.0016$) were affected by site, and therefore, results are presented by site (Table 2).

Serum total carotenoids had an overall mean concentration of 2.48 ± 1.2 μmol/L (median 2.41 μmol/L) and did not differ by site. A common reference range for serum carotene is 0.9–3.7 μmol/L (28), which would encompass the overall mean. Sixteen samples (13%) had total carotenoid concentrations > 3.7 μmol/L, which were considered hypercarotenemic.

Fasting serum retinyl esters and ratio of retinol to retinol-binding protein

Retinyl oleate, palmitate, and stearate were identified in serum extracts (n = 123). Other esters were present but sometimes overlapped with other unidentified compounds, which could have been carotenoids that might have been detectable at 325 nm at the high concentrations in many of the samples. Retinyl esters were not statistically significantly affected by age, sex, BMI, CRP, or AGP; however, the retinyl ester percentage of serum total VA (P = 0.0009) was affected by site, and therefore, results are presented by site (Table 2). In all participants, 16% of subjects had retinyl esters >5% of serum total VA, whereas 2% of subjects had retinyl esters >10% of serum total VA. In line with higher TBSs, retinyl esters in village B were also statistically significantly higher when based on total retinol equivalents in the serum, and the third quartile value (75th percentile) included the lower cutoff of >5% (Table 2). SR and RBP concentrations did not differ by site. The ratio of retinol to RBP was very close to 1.0 and did not differ by site. As expected, but with low r^2 values, RBP was negatively

correlated with CRP ($P = 0.035$, $r^2 = 0.06$) and AGP ($P = 0.010$, $r^2 = 0.09$).

Serum ALT activity and pyridoxal-5′-phosphate

The liver enzyme ALT was evaluated in serum to determine whether liver damage was present because of the hypervitaminotic state of some children. ALT activity was below normal (range: 0.83–11.4 U/L), and only one child tested had normal activity (10-40 U/L). ALT activity was not related to any other factors evaluated. Because ALT activity was low, PLP concentrations were determined and were also below normal; 79% of children had serum concentrations <20 nmol/L, which is the suggested deficiency cutoff (29), and 29% of values were in the extremely low range of <10 nmol/L. Although the r^2 was low ($r^2 = 0.06$), PLP was statistically significantly negatively associated with CRP ($P = 0.020$).

DISCUSSION

This study reports supporting biomarkers of VA status in a group of children with a large percentage diagnosed with hypervitaminosis A by using an RID methodology (7). The children had relatively low weight- and height-for-age, and some children had subclinical malaria. Provitamin A carotenoid concentrations before the dietary intervention were higher than in most other global populations, including in developed countries with minimal VA deficiency (**Table 3**). Fasting retinyl esters were slightly elevated in some children, and the retinol-to-RBP ratio was 1.0. Although we assayed ALT activity to assess potential liver involvement,

TABLE 3
Serum carotenoid concentrations in apparently healthy children from various regions of the world[1]

Country	Age, y	n	Notes	Retinol, μmol/L	β-Carotene, μmol/L	α-Carotene, μmol/L	β-Cryptoxanthin, μmol/L	Lutein, μmol/L	Lycopene, μmol/L	Reference
Zambia	5–7	123	—	0.98	0.76	0.62	0.10	0.86	0.30	Current
Belize	3–9	493	—	—	0.21	0.093	0.15	0.23+z	0.11	Apgar and Gunter (30)
China	0.5–2	254	—	0.96	0.056	0.003	0.027	0.22+z	—	Fan et al. (31)
Germany	—	49	Native German	0.99[2]	0.125[2]	0.026[2]	0.090[2]	0.093+z[2]	0.017[2]	Rühl et al. (32)
	—	32	Turkish immigrants, well-adapted	0.90[2]	0.093[2]	0.015[2]	0.061[2]	0.11+z[2]	0.017[2]	
	—	41	Turkish immigrants, weakly adapted	0.94[2]	0.071[2]	0.011[2]	0.056[2]	0.10+z[2]	0.019[2]	
Hungary	3.1–17.5	29	Noninfectious	1.17	0.16	0.049	0.16	0.12+z	<0.007	Cser et al. (33)
India	2–11	50	Nonmalarial	1.10[2]	0.31[2]	0.035[2]	0.12[2]	0.42[2]	0.068[2]	Das et al. (34)
Japan	10–11	216	—	—	0.41	0.11	0.19	0.45+z	0.18	Okuda et al. (35)
Marshall Islands	1–6	189	SR <0.7 μmol/L	—	0.011[3]	0.003[3]	0.023[3]	0.044+z[3]	0.007	Gamble et al. (36)
		89	SR >0.7 μmol/L	—	0.023[3]	0.006[3]	0.034[3]	0.052+z[3]	0.012	
Nigeria	0.7–8	19	Nonmalarial	0.78	1.29	—	—	—	—	Adelekan et al. (37)
Philippines	6.8–13.2	27	Baseline	0.68	0.13	0.02	0.06	0.50+z	0.05	Ribaya-Mercado et al. (5)
			After 12 wk F/V	1.06	0.66	0.15	0.19	0.47+z	0.25	
Philippines	9–12	116	Baseline	0.87	0.23	0.03	0.07	0.22	—	Ribaya-Mercado et al. (12, 14)
			After 9 wk F/V	0.90	1.17	0.57	0.12	1.29	—	
Senegal	2–4	281	—	—	0.16	0.030	0.020	0.46+z	0.070	Rankins et al. (38)
Sri Lanka	0.5–4.8	35	Individuals with carotenodermia	1.35	0.61	0.63	0.26	—	—	Wageesha et al. (39)
United States	6–7	839	NHANES III	—	0.34	0.075	0.21	0.34+z	0.46	Ford et al. (13)
United States	0.4–6	77	—	1.09	0.19[2]	0.05[2]	—	—	0.15[2]	Spannaus-Martin et al. (40)

[1] All values are means unless otherwise indicated. After Zambia (current study), countries are listed in alphabetical order. F/V, fruit and vegetable intervention; SR serum retinol; +z, zeaxanthin included in the published lutein value.

[2] Median.

[3] Geometric mean.

a high prevalence of vitamin B-6 deficiency was discovered, which likely interfered with the ALT activity.

Inferences from serum carotenoids consistent with high liver VA stores

Aside from dietary intake, serum carotenoid concentrations can be altered by a number of factors including VA status, the transcriptional regulation of or genetic variations in genes related to carotenoid metabolism, and BMI. VA status is likely the most important factor that influenced BCO1 activity (41). A negative-feedback system induced by retinoic acid reduced β-carotene intestinal absorption and cleavage proteins (19) and led to less-efficient β-carotene conversion in rats that consumed a high-VA diet (17), which explains the inverse relation between VA status and provitamin A conversion in humans (5). Although serum carotenoid increases were not analyzed by VA status in the study of Ribaya-Mercado et al. (5), TBSs and serum carotenoids increased during the intervention, indicating that, while under negative feedback of VA status, carotenoids were still being absorbed intact in most subjects despite a likely downregulation of carotenoid transporter scavenger receptor-B1. The observations of adequate VA intakes including provitamin A sources (23), elevated TBSs (7), and high serum carotenoids in Zambian children are consistent with these hypotheses.

Polymorphisms in BCO1 are associated with elevated fasting β-carotene concentrations (42), and a lower conversion efficiency of β-carotene, albeit at pharmacologic doses (43). Although statistically significant, a polymorphism associated with a higher β-carotene concentration only explained 1.9% of the variance at the population level (42), and other variations in genes related to the absorption, transport, and cleavage of carotenoids are likely involved in the individual variation in response to dietary carotenoids (16) as well as their transcription factors (18).

BMI was negatively correlated with all serum carotenoids except lycopene in the most-comprehensive survey of serum carotenoid concentrations in US children as part of the NHANES III (13). Responses of plasma carotenoids to a fruit and vegetable intervention were also inversely related to BMI (14). Zambian children in this study had low BMI-for-age z scores, which may be associated with higher serum carotenoid concentrations. However, even when NHANES III data were stratified by lowest BMI category ($\leq$15th percentile), Zambian children mean values were still above the 95th percentile for serum α-carotene, β-carotene, and lutein (13).

A comprehensive comparison of published serum carotenoid and retinol concentrations in apparently healthy children was conducted (Table 3); children with an infection or active malaria were excluded, and control groups are presented. For provitamin

A carotenoids, Zambian children had much-higher concentrations of α-carotene and β-carotene than those of all other nonintervened groups except for Nigerian children who were reported to have been consuming red palm oil (37), which contains large amounts of α-carotene and β-carotene (44). α-Carotene is not well distributed in vegetables but is present in pumpkin (9), which is consumed in Zambia (23). β-Cryptoxanthin concentrations were comparable to those of other groups but lower than for US children (Table 3). For non–provitamin A, lutein concentrations were higher than those of other nonintervened groups, and lycopene concentrations were higher than those of all groups except of US children (13). These findings reflect the availability of oranges and tomato-based products in the United States. Oranges are not common in this part of Zambia, and tomatoes are eaten freshly cooked and not concentrated. The only groups of children with carotenoid concentrations close to those of this Zambian cohort are those in individuals who consumed high fruit and vegetable regimens that dramatically increased serum carotenoids (5, 12, 14) and in subjects with carotenodermia that was due to excessive ingestion of carrot, pumpkin, and papaya (39).

Differences existed by site in serum carotenoid concentrations. Village B stood out from the others as having the highest α-carotene and lycopene concentrations but lowest lutein concentrations, which likely reflected greater consumption of pumpkin and tomatoes and less intake of leafy green vegetables relative to other sites. All of these plant-source foods would have been available during this harvest and early postharvest season (23).

Inferences from retinyl ester distribution and retinol:RBP

Evidence from animal and human studies suggested that serum retinyl esters are a potential biomarker for hypervitaminosis A. In hypervitaminotic rhesus monkeys, serum retinyl esters as a percentage of the total ranged from 5.5% to 23% for animals experiencing hypertrophy and hyperplasia of liver stellate cells (45, 46) but with normal SR concentrations (i.e., 1.21 ± 0.28 μmol/L) (46). In 2 groups of postmenopausal women, serum retinyl esters were not considered elevated ($2.26 \pm 1.39\%$ and $2.45 \pm 1.30\%$) despite dietary intakes that were 2 times the current RDA of 700 μg retinol activity equivalents/d (47). Therefore, we suggest that 5% retinyl esters of total VA, which is more than twice the mean of these healthy women with high intakes, may be a more-useful cutoff in fasting blood samples to infer potential hypervitaminosis A in children than is the 10% cutoff when more-quantitative methods, such as RID and liver biopsy, are not available.

RBP is used as a surrogate for SR concentrations, which were not elevated. Seventeen percent of subjects were misdiagnosed with VA deficiency on the basis of an SR cutoff <0.7 μmol/L (7). In this study, the retinol-to-RBP molar ratio was essentially 1.0, suggesting that RBP was highly saturated; free retinol was circulating unbound to RBP, which is a concern in hypervitaminotic states (22), and apo-RBP was not released into the serum from the liver, which can occur in VA deficiency (48, 49). Furthermore, considering that our children were not overweight, apo-RBP was not shown circulating without its ligand, which is characteristic of excess adipose tissue (50).

Paradoxical response of ALT and its explanation by impaired vitamin B-6 status

Serum ALT activity was assayed to determine whether elevated VA stores resulted in hepatotoxicity (51); however, values were below normal. PLP is a cofactor for ALT; therefore, vitamin B-6 deficiency interferes with ALT-activity assessment. Although inflammation was reportedly associated with lower PLP values (52), as observed here, the observation of extremely low PLP concentrations (29.3% of values <10 nmol/L) and a very-high incidence (79%) of serum PLP concentrations <20 nmol/L strongly indicated widespread vitamin B-6 deficiency in these children. Functional biomarkers of vitamin B-6 status (53, 54) are needed to confirm and extend these findings.

Maize is the Zambian staple food, and the common practice of refining maize and removing the nutritious germ and hull to improve consistency impacts intakes of vitamin B-6 and other nutrients (55). Although fish is consumed in this area, consumption is likely $\sim$50 g one or 2 times/wk (23). Other animal source foods are expensive or scarce in rural Zambia. A modification to use whole-grain maize instead of refined could impact human nutrient intake and merits additional investigation. Site differences were noted, and children from village C had the lowest PLP concentrations of all sites, and villages closest to the main road (i.e., villages A and B) had the highest concentrations, which possibly reflected greater access to foods containing vitamin B-6.

In conclusion, although this study used sophisticated RID methods to estimate TBSs, which requires the use of mass spectrometry, less-technical methods were used to support the original diagnosis of a high degree of hypervitaminosis A in these children. Carotenoid and retinyl ester profiles can be performed with gradient HPLC. To have high sensitivity to detect minor retinyl esters in addition to palmitate, we analyzed the carotenoid and retinyl ester profiles on different systems. A method could be developed to run both profiles simultaneously especially if retinyl palmitate is targeted. The findings of hypercarotenemia, saturated RBP, and elevated retinyl esters in some of the children support excessive stores of VA in this community.

alliance of agriculture and nutrition research institutions working to increase the micronutrient density of staple food crops through biofortification. The views expressed in this article do not necessarily reflect those of HarvestPlus. None of the authors reported a conflict of interest related to the study.

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