RBP-EIA:

Examination of Venous and Capillary Blood Specimens for Assessment of Vitamin A Deficiency

PATH
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May 2005

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Acknowledgments

The authors would like to thank Deborah Lans, Frances Davidson, Carol Levin, Dean Garrett, Dan Chang, and Jeff Huentelman for their input and assistance in supporting this evaluation. In addition, Napapron Riabroy and Premmin Srisakda conducted laboratory analysis of the samples in Thailand; and Napapron Riabroy, Premmin Srisakda, Kandavasee Maleevong, Srirat Nuamdee, Wimolthip Krangpanich, Achara Pornsateinkul, and Piyanan Padmuang supported field enumeration.

Support for this evaluation was made possible by the United States Agency for International Development under the Health Tech IV project, Cooperative Agreement Number SPH-A-00-01-00005, managed by PATH.

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Introduction and Background

Vitamin A deficiency (VAD) is a significant public health problem in many countries and is the leading cause of preventable blindness throughout the world, particularly among children under five years of age. Recent estimates have shown that, globally, over 140 million people are affected by VAD, often leading to impaired immunity, xerophthalmia, and even death.[1] Every year VAD directly contributes to blindness in at least half a million children and also indirectly contributes to the deaths of an estimated three million children worldwide.

There are several cost-effective interventions which are being implemented in many countries to address VAD including food fortification, dietary improvement, and pharmacologic supplementation. In order for countries to identify the most appropriate intervention strategy or mix of strategies to implement, there is the need for reliable and accurate information on the magnitude and distribution of VAD in populations. Indeed, such information is also important to track changes in vitamin A (VA) status following the introduction of interventions and to ensure that all segments of the population are being reached and are being impacted by program efforts. Although assessment of VAD and VA status in populations is extremely important, it is not commonly done because current analytical methods are expensive, unreliable, and not appropriate in low-resource settings where VAD is most endemic.[2] As a result there is a scarcity of robust epidemiological data on the prevalence of VAD in many countries, and, where interventions are in place, it has often not been possible to evaluate program effectiveness.

The World Health Organization (WHO), UNICEF, and the International Vitamin A Consultative Group (IVACG) have proposed a series of clinical and biochemical indicators to identify and monitor VAD in populations and to assess VA status.[3] In general, clinical indicators such as night blindness and other signs of xerophthalmia are rare and require very large sample sizes. These parameters are also prone to measurement variability and misclassification.[4,5] The measurement of clinical xerophthalmia and ocular manifestations of VAD is only appropriate in populations with severe deficiency as these indicators are not sensitive in detecting subclinical or marginal levels of VAD. As an alternative to clinical parameters, biochemical indicators have also been recommended to identify populations with marginal VAD as well as compromised VA status, which itself has been found to be associated with an increased risk of mortality.[6,7]

The principle biochemical indicator of VA status recommended for international use has been serum or plasma retinol.[8] As such, retinol is the most commonly employed and accepted biochemical indicator of VAD and is usually assessed by high-performance liquid chromatography (HPLC), fluorometry, or UV-spectrophotometry. Although fluorometry and UV-spectrophotometry are less expensive and require less sophisticated equipment than HPLC, they are not as accurate in estimating retinol concentrations. While

recognized as the gold standard, the measurement of retinol by HPLC can be logistically complex requiring a large sample volume and careful specimen collection, handling, and storage. Although numerous laboratories in both industrialized and developing countries perform serum retinol analysis using HPLC, their proficiency varies. A proficiency test for laboratories measuring retinol and carotenoids reported that only 7 out of the 16 participating developing-country laboratories were able to measure serum retinol to an acceptable standard.[9] It should be noted that for many years serum levels of carotenoids and retinol have been underestimated in many countries when measured by HPLC.[10]

Recently, retinol-binding protein (RBP) has been reported to correlate well with serum retinol in different population groups and has been found to be proficient in estimating the prevalence of VAD among populations in several small field studies.[11,12] One advantage of RBP is that it is a protein and can be analyzed by immunological diagnostic tests using antibodies to detect and quantify the RBP concentration in serum. A competitive enzyme immunoassay (EIA) has been developed which is significantly less expensive than HPLC and requires only standard equipment, such as a standard micro well plate reader which may be available in many laboratories in developing countries currently doing EIA-based testing.

In addition to the utility of RBP as a surrogate indictor of retinol and as an appropriate indicator of VAD, another important factor to consider is the different biological specimens that may be collected and subjected to analysis for the assessment of VAD in population-based surveys. Most often the assessment of retinol has been done using HPLC, which requires a large volume of serum separated from venous blood. One advantage of the retinol binding protein enzyme immunoassay (RBP-EIA) is that it requires a relatively small volume of serum, which could conceivably come from capillary blood collected by finger prick.

The collection of blood by venipuncture can be difficult, and many people in communities in developing countries may be uncomfortable with this practice. Furthermore, there are field and safety constraints that limit the feasibility of venous blood collection. There has been a great deal of interest in assessing VAD using capillary blood, either stored in microcapillary tubes or as dried blood spots (DBS) on a filter paper matrix.[13,14,15] However, there are no published studies that specifically examine the biological equivalence between different parameters of VAD from venous blood and from capillary blood.

While the potential for assessing VAD from serum samples collected from capillary blood is promising, their correspondence and relationship to an established reference standard, such as retinol from venous serum by HPLC, need to be demonstrated. Consequently this study aimed to evaluate the relationship between different methods for the assessment of VA status and for the classification of VAD at the population level.

The primary objectives of the study were:

- To evaluate the screening proficiency of RBP relative to retinol in estimating the prevalence of VAD at the population level.
- To evaluate the feasibility of capillary blood for use as a biological specimen to assess VA status and estimate VAD in populations.

Methods

The study was carried out in two remote Districts of Chiang Mai, Thailand: Mae Cham and Om Koi Districts. Data collection took place over a one-week period between September 3–10, 2004. In 2000–2001, there were reported cases of xerophthalmia among infants and young children in both districts and it was expected that 10% to15% of the preschool children would have marginal serum retinol levels. While these two districts are included in the Thailand National VAD Control program, the data enumeration for the study was planned to ensure that none of the children in these two districts had received a VA supplement within the previous five to six months. Matched panels of venous and capillary blood were taken from a cohort of preschool children. From these, four parameters of VA status were estimated based on indicator and specimen type as summarized below.

Table 1. Four Parameters of Vitamin A Status Measured

Parameter	Indicator	Specimen	Lab Method
1	Retinol	Venous—serum	HPLC
2	Retinol	Capillary—serum	HPLC
3	RBP	Venous—serum	EIA
4	RBP	Capillary—serum	EIA

In addition to the four parameters of VA status, C-reactive protein (CRP) and α-1-glycoprotein (AGP) were analyzed from venous blood to control for inflammatory disease.[16,17] Finally, hemoglobin levels of the children were measured in the field directly from capillary blood using a portable Hemo-CueTM device (HemoCueTM AB, Angelholm, Sweden).

The sample size for the study was estimated to be 200 children in order to characterize the distribution of biochemical indicators of VA and to analyze the correspondence between

indicators using standard screening parameters. The sample size was calculated based on an expected screening level of 85% with absolute precision of +5% and 95% confidence.1 Serum retinol from venous blood analyzed by HPLC was taken as the gold reference and all other parameters of VAD were compared to this standard indicator.

The target age group for the study was preschool children between the ages of 12 and 60 months (although a small number of older children were included) as this is the group with the highest risk of morbidity and mortality associated with VAD. This group also contains the age cohort most often targeted for VA supplementation activities and in which assessment of VAD is critical. The sample was divided between the two districts of Mae Cham and Om Koi, and 100 children were recruited for each area. Local health workers developed a list of all children in the target age group and a sample was selected at random.

Ethical and human subjects clearance for the study were obtained from the Scientific Committee of the Institute of Nutrition at Mahidol University (INMU, Nakhon Pathom, Thailand) and from the Program for Appropriate Technology in Health (PATH, Seattle, WA). Consent for the study was also obtained from Provincial and District Health authorities following a series of meetings with representatives of INMU and the Nutrition Division of the Ministry of Health. Local government health officials from the District Health Office and the Primary Care Unit approached the mothers of the children, explained the objectives of the study, and requested consent for a physical check-up and the participation of the children in the study. Before the start of the physical examination, written consent was requested from all parents/guardians who agreed for their child to participate. The parents were asked to have their children gather at a central data collection location where data enumeration, including the collection of biological specimens, was handled systematically and in a controlled manner.

A questionnaire was administered to collect basic demographic data of all participating children, including age and gender. Clinical examinations focusing on acute diarrhea and respiratory illness were undertaken by trained medical personnel to ascertain current status of infectious morbidity. After the administration of the questionnaire and the physical

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[1] The sample size calculation was based on the following formula, where we assumed a screening index (sensitivity and specificity) of 85%, a confidence level ($Z_{1-\alpha/2}$) of 95% (or 1.96), and a desired precision of 5%.

$$Sn \pm Z_{1-\alpha/2}\sqrt{\frac{Sn(1-Sn)}{n_a}}$$

$$Sp \pm Z_{1-\alpha/2}\sqrt{\frac{Sp(1-Sp)}{n_n}}$$

examination, each child and the child's mother were brought into a private blood collection room where blood samples were taken.

Nurses first drew venous blood with a Vacutainer Blood Collection Set or Sarstedt Multi-Fly Set (Becton Dickinson and Co., Franklin Lakes, NJ) or Sarstedt Multi-Fly Set (SARSTEDT AG & Co., Numbrecht, Germany). The volume of venous blood collected was approximately 2 mL from each child. Each tube was labeled with an individual identification code. The nurse then collected capillary blood by finger puncture using clean, new, disposable lancets (BD Genie). Free-flowing capillary blood was collected after wiping away the first blood drop, with the second blood drop used to fill a cuvette for analysis of hemoglobin in a portable HemoCueTM device. In addition an average of 5 to 6 capillary tubes of blood were collected from each child. In some cases a second finger puncture was required in order to retrieve sufficient volume of capillary blood. The capillary tubes (VITREX BRIS MicroHaematocrit Tubes, REF 160231, ISO 12772, Plain, Nonheparinized from MODULOHM A/S, Herlev, Denmark) were plugged with clay, and tape was placed on these with the individual codes. The blood was allowed to clot for 40 minutes and then centrifuged. Venous blood was centrifuged with a Compact Lab Centrifuge (Labnet, Model Z150A) at 3500 rpm for 15 minutes, while capillary blood was centrifuged with a Hettich 2010 or DAMON/IEC (Model MB) for 7 minutes. Serum from venous blood was separated into two amber microtubes, the first containing at least 0.5 mL and the second containing 0.2 mL.

Serum from capillary blood was collected by cutting the capillary tubes with a file, and two aliquots of at least 50 µL of serum were transferred to individual polymerase chain reaction (PCR) tubes. At the end of each day of data collection the tubes were labeled with code numbers, placed in storage boxes, and then placed in ice pack boxes and transported to Mae Cham Hospital or Om Koi Hospital. These samples were maintained in the hospital's freezer at –20oC. When data collection was completed, all specimens were shipped frozen to the laboratory at INMU for analysis. RBP was analyzed from serum of both specimen types using the PATH RBP-EIA test (Scimedx, Denville, NJ). Retinol was analyzed from both capillary and venous serum by conventional HPLC, while CRP and AGP were measured from serum of the venous specimens by RID (Kent Laboratories, Bellingham, WA). All analytes were analyzed within three weeks of data collection in the field.

Capillary and venous serum samples were analyzed for both retinol and RBP. First, both specimens and calibrator sera were diluted 1:25 in assay buffer in a low protein-binding micro well plate. The specimens and calibrator sera were next mixed by reverse pipetting (Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY) and transferred to test wells in plastic low protein-binding micro well plates (Evergreen Medical Products, Bainbridge Island, WA). One hundred microliters of monoclonal anti-RBP antibody conjugated to horseradish peroxidase enzyme were then immediately added to each sample test well.

The plates were incubated for 15 minutes at room temperature (18°C to 25 °C); after 10 minutes the sample wells were mixed by gently tapping the plate frame.

At the end of the 15-minute incubation period, test plates were inverted and emptied by flicking the contents into a sink. The test wells were then washed 5 times using wash buffer. Immediately following the final wash, 200 µL of 3, 3′5, 5′ tetramethylbenzidine (TMB, Moss Ultra–sensitive, Hanover, MD) containing hydrogen peroxide substrate were added to each well. The plates were incubated at room temperature (18°C to 25 °C) for another 10 minutes, mixed briefly at 5 minutes and again near the end of the incubation by gently tapping the plate frame. Following this incubation, 100 µL of stop solution was added to all of the test wells. The plates were immediately read using the EIA plate reader (Sunrise, Tecan Austria GmbH, Salzburg, Austria) fitted with a 450 nm filter, and the resulting optical densities were recorded using Magellan software version 3.12 (Tecan, Austria). The results from the analysis were compared to values obtained from the calibrator sera by linear regression software or by graphing the RBP concentration versus the optical density obtained from the calibrator sera.

The samples were thawed, an aliquot of 50 µL was extracted in hexane, and 20 µL were injected by an auto-sampler (Waters 717plus) onto an analytical column and analyzed by reverse-phase HPLC (Waters Corp., Milford, MA). Retinol was detected using a programmable UV/VIS detector (Waters 486, Waters Corp., Milford, MA) that was set at 325 nm. Retinol was separated using a ResolveTM C18 analytical column (150.0 mm X 3.9 mm diameter, Waters Corp., Milford, MA) containing 5 µm particles protected with a C18 guard column 2.0 mm X 20.0 mm (Upchurch Scientific, Inc., Oak Harbor, WA). Isocratic analyses were performed at a flow rate of 0.7 mL/min with a mobile phase of absolute methanol that was delivered by a Waters 515 dual-head pump (Waters Corp., Milford, MA). The data for each determination was transmitted, stored, and processed by a personal computer using Millennium Workstation software (version 3.05.01, Waters Corp., Milford, MA).

Data Analysis

Four parameters of VA status were calculated. Data were examined for normal distribution characteristics using the Kolmogorov-Smirnov test. Linear correlations were plotted between the three test parameters of VA status (retinol-capillary, RBP-venous, and RBP-capillary) against the reference standard retinol-venous. In addition, Bland-Altman plots were generated to assess the agreement between the different methods in measuring VA status. To classify VAD, the cutoff point of 0.70 µmol/L as recommended by WHO and IVACG was used for all parameters. In addition, a receiver operating characteristic (ROC) curve was plotted to determine the optimal correspondence between different cutoff criteria for RBP from venous blood in classifying VAD as compared to retinol < 0.70 µmol/L. The ROC provides a graphic representation of the balance between

sensitivity and specificity of a diagnostic tool relative to a reference standard. That is, any increase in sensitivity will be accompanied by a decrease in specificity. The closer the curve follows the left vertical axis and then the top horizontal axis of the ROC space, the more accurate the test. In turn, the area under the curve is a measure of the overall accuracy of the diagnostic test parameter.

To assess inflammation, cutoff points were used for CRP (> 5.0 mg/L) and for AGP (> 1.0 g/L) as recommended by Thurnham and Clewes. Based on these criteria the status of inflammation was classified to reflect the stage of infection as (1) normal—no elevated acute-phase proteins; (2) incubation—elevated CRP, but normal AGP; (3) early convalescence—both acute-phase proteins elevated; and (4) late convalescence—elevated AGP but normal CRP.[18] For the classification of anemia, the cutoff point < 11 g Hb/dl was designated as recommended by WHO for preschool children.

Differences in prevalence of VAD based on the different indicators were measured by Pearson's χ^2 test. Differences in the distribution of indicators of VA status between groups of children with different inflammation status were analyzed using Student's paired t-test. Linear regression models were plotted to establish the correlation between the different parameters of VA status. Statistical analysis was carried out using SPSS 12.0 (SPSS, Chicago, IL). The sensitivity, specificity, positive predictive value, and negative predictive value were calculated relative to retinol from venous blood using the cutoff point of < 0.70 µmol/L taken to reflect the reference standard for the classification of VAD.

Results

Of the total sample of 200 children, 198 were given parental consent to participate in the study. Of the 198, venous blood could not be collected from 2 children, while the capillary blood from another child was not available as the capillary tube broke when it was being centrifuged in the field. There were, therefore, totals of 196 venous samples and 195 capillary blood specimens collected, and a matched panel was available for a cohort of 195 children. There was sufficient volume of both capillary blood and venous blood from all 195 children to undertake all lab analyses as per the study protocol.

Table 2 provides general demographic data of the study population. There was equal representation from both of the study districts, and the gender balance was also equivalent. There was an effort to ensure that preschool children between 12 and 59 months of age were included in the cohort, and this was realized with some 93% of all children falling in this range, including some 21.7% under two years of age. It was possible to collect an adequate volume of capillary blood from these younger children.

Table 2. Basic Characteristics of Study Population

Parameter	n	%
District		
Om Koi	100	50.5
Mae Cham	98	49.5
Gender		
Boys	102	51.5
Girls	96	48.5
Age		
12–24 months	43	21.7
24–36 months	42	21.2
36–48 months	54	27.3
48–60 months	45	22.7
> 60 months	14	7.1

The correlation between retinol from venous blood and the three other parameters of VA status are presented in Appendix 1. While the focus of this study was to identify which indicator would be able to most efficiently distinguish between VAD and adequate VA status among populations, the correlations do provide some impression of the linear correspondence between the three test parameters and retinol from venous blood. The best fit was noted between RBP-venous and retinol-venous estimates (R2 = 0.63), although there was good linear correlation between retinol-venous and the two other parameters, RBP-capillary (R2 = 0.60) and retinol-capillary (R2 = 0.59). The agreement among methods for all three test parameters was strongest at the lower tail of the distribution corresponding to poor VA status, while the concurrence was less pronounced at the upper tail of the distribution, in part since the RBP test was optimized at the lower end to best detect deficiency.

The linear association between the different methods is seen further in evaluating Bland-Altman plots, which are also presented in Appendix 1. These plots are helpful in examining how well two methods correspond by plotting the average of the two measures (on the horizontal axis) against the differences between the two measures (on the vertical axis). These plots are most applicable when considering the agreement between methods along the entire range of VA status, not only the lower tail of the distribution which is the focus of VAD assessment.

An ROC curve was plotted to assess the most appropriate cutoff point for RBP relative to retinol with < 0.70 µmol/L retinol taken as the reference criteria for VAD. While there has been an assumption that the same cutoff point used to classify VAD based on retinol be used for RBP based on the assumption that there is a 1:1 molar relationship between these compounds, other studies have noted that a higher cutoff point achieves greater

proficiency. Indeed, in this study the ROC analysis suggested that a cutoff point of < 0.825 µmol RBP/L had the most optimal screening proficiency (area under curve = 0.011) relative to retinol < 0.70 µmol. Extensive irradiation of serum samples had no effect on the assays estimate of RBP level indicating that the monoclonal antibody reacted equally well with apo- and holo-RBP in the assay (data not shown).

Table 3 presents data on the status of VA deficiency, anemia, and inflammation in the study population. The mean VA levels were slightly lower for retinol than for RBP with virtually no difference between the capillary and venous blood samples for each parameter. All four parameters followed normal distributions and had similar variance estimates. The prevalence of VAD based on serum retinol from venous blood using the cutoff point of < 0.70 µmol/L was 7.2% (95% CI 3.6,10.8), while the prevalence was slightly higher when using retinol from capillary blood (7.7%, 95% CI 3.9,11.4). Using a cutoff point for RBP of 0.825 µmol/L as suggested by the ROC analysis, the prevalence of VAD was 7.7% from venous blood (95% CI 3.9,11.4) and 9.2% from capillary blood (95% CI 5.1,13.2). The differences in VAD prevalence between the four parameters did not reach statistical significance.

Table 4 outlines the screening proficiency of the three VA parameters against retinol analyzed from venous blood. Not surprisingly, given the low prevalence of VAD observed in the study population, the specificity of all parameters was very high, all above 96.5%. For retinol from capillary blood, the sensitivity was 71.4%. Using a cutoff point of 0.825 µmol RBP/L for RBP, the sensitivity of the RBP from venous blood was 92.9% while the sensitivity of capillary blood was 85.7%. For purposes of population assessment, the proficiency of all three test parameters seemed to perform as well as one another in screening for VAD relative to retinol from venous blood.

There was a significant association between CRP and AGP. There was a 20-fold increased probability of a child having elevated CRP if he also had an elevated AGP. In fact, of the 19 children with elevated CRP, 17 also had high AGP levels indicating that most children had advanced from the incubation stage of infection to early convalescence. Table 5 presents data showing the association between inflammation and VAD.

Table 3. Biological Parameters of Study Population

Parameter	Mean	SD	Cutoff	%	95% CI
Retinol-venous (µmol/L)	1.063	0.26	< 0.70 µmol/L	7.2	3.6,10.8
Retinol-capillary (µmol/L)	1.076	0.29	< 0.70 µmol/L	7.7	3.9,11.4
RBP-venous (µmol/L)	1.121	0.22	< 0.82 µmol/L	7.7	3.9,11.4
RBP-capillary (µmol/L)	1.118	0.23	< 0.82 µmol/L	9.2	5.1,13.2
Hb (g/dl)	11.65	1.43	< 11 g/dl	28.4	22.1,34.7
AGP (g/L)	0.916	0.33	> 1.0 g/L	24.0	18.0,30.0
CRP (mg/L)			> 5 mg/L	9.7	5.6,13.8
Inflammation status [a]					
Healthy				66.3	59.7,72.9
Incubation				1.0	0.1,3.6
Early convalescence				8.7	5.1,13.5
Late convalescence				24.0	18.2,30.6

[a] Healthy—No raised acute-phase proteins
Incubation—CRP > 5 mg/L, AGP ≤ 1.0 g/L
Early convalescence—CRP > 5 mg/L, AGP > 1.0 g/L
Late convalescence—CRP ≤ 5 mg/L, AGP > 1.0 g/L

Table 4. Proficiency in Estimating VAD by Different VA Parameters: Controlling for Inflammation Status (All comparisons are relative to retinol-venous < 0.70 µmol/L.)

		All children (n = 196)		Early convalescence (n = 17)		Late convalescence (n = 47)	
Parameter	**Cutoff**	**Se**	**Sp**	**Se**	**Sp**	**Se**	**Sp**
Retinol-cap	< 0.70 µmol/L	71.4	97.2	100.0	76.9	66.7	100.0
RBP-venous	< 0.825 µmol/L	92.9	98.9	75.0	92.3	100.0	100.0
RBP-cap	< 0.825 µmol/L	85.7	96.7	75.0	92.3	100.0	97.7

There were no differences in the estimates of VAD within each cohort of children stratified by inflammation status. That is, all four parameters responded in the same manner to different stages of infection. There was a dramatically higher level of VAD among those children in the early convalescent stage of infection from all parameters of VA status than children with no infection or in the late convalescent stage. However the

prevalence of VAD estimated by retinol from venous blood among children in the early convalescent stage of illness (41.2%; 95% CI 18.4,67.1) was significantly higher than among children with no inflammation (4.6%; 95% CI 1.7,9.8) and among children in later convalescence (4.3%, 95% CI 0.5,14.5).

Table 5. Prevalence of VAD According to Different Classifications: Stratified by Inflammation Parameters (exact 95% confidence intervals based on binomial distribution[a])

	Prevalence of VAD (95% CI) in groups based on inflammation status			
VAD parameter	Early convalescence[b] (n = 17)	Late convalescence[c] (n = 47)	No inflammation[d] (n = 130)	All children (n = 196)
Retinol-venous < 0.70 µmol/L	23.5 (6.8,48.9)	6.4 (1.3,17.5)	4.6 (1.7,9.8)	7.1 (4.0,11.7)
Retinol-capillary < 0.70 µmol/L	41.2 (18.4,67.1)	4.3 (0.5,14.5)	4.6 (1.7,9.8)	7.7 (4.4,12.3)
RBP-venous < 0.825 µmol/L	23.5 (6.8,48.9)	6.4 (1.3,17.5)	5.4 (2.2,10.8)	7.7 (4.4,12.3)
RBP-capillary < 0.825 µmol/L	23.5 (6.8,48.9)	8.5 (2.4,20.4)	6.9 (3.2, 12.7)	9.2 (5.5,14.1)

[a] Brown, L.D., T.T. Cai, and A. Das Gupta. 2001. Interval estimation for a binomial proportion, *Statistical Science* 16: 101-133.
[b] Elevated CRP (> 5.0 mg/L) and elevated AGP (> 1.0 g/L).
[c] Elevated CRP (> 5.0 mg/L), normal AGP.
[d] No elevated acute phase proteins.

There were no differences between the mean retinol values from venous blood and capillary blood for all groups of children when accounting for infection (Table 6). The mean values were lower for those children in the early convalescence stage, but both venous and capillary blood followed identical patterns.

There were also no differences between the mean RBP values from capillary and venous blood among all children, as well as when stratified by inflammation status. In fact, the correspondence between the RBP from venous and capillary blood seemed to be even stronger than seen with retinol. As expected, the overall mean levels of RBP were slightly higher than those for retinol.

Table 6. Comparison Between Methods of VAD Assessment: Stratified by Inflammation Status—Retinol

	Retinol (µmol/L)				
Parameter	**Venous**		**Capillary**		
	Mean	**SD**	**Mean**	**SD**	**p-value**
All children (n = 196)	1.06	0.26	1.07	0.29	.356
Early convalescence (n = 17)	0.86	0.25	0.80	0.28	.148
Late convalescence (n = 47)	1.11	0.31	1.12	0.33	.672
No inflammation (n = 130)	1.08	0.23	1.10	0.25	.232

Paired T-Test * p < 0.05, ** p < 0.01, *** p < 0.001

Table 7. Comparison Between Methods of VAD Assessment: Stratified by Inflammation Status—RBP

	RBP (µmol/L)				
Parameter	**Venous**		**Capillary**		
	Mean	**SD**	**Mean**	**SD**	**p-value**
All children (n = 196)	1.12	0.22	1.12	0.23	.873
Early convalescence (n = 17)	0.95	0.21	0.94	0.21	.677
Late convalescence (n = 47)	1.13	0.22	1.13	0.24	.823
No inflammation (n = 130)	1.14	0.21	1.14	0.22	.806

Paired T-Test * p < 0.05, ** p < 0.01, *** p < 0.001

Conclusions and Discussion

This study provided the first evidence of the biological comparability between serum retinol levels estimated from venous blood and capillary blood. This is a critical observation as it provides empirical evidence that this important indicator of VA status may be measured from capillary blood, which has important implications for nonclinical

population-based research. Finger prick sampling collection is relatively painless and noninvasive, and capillary blood sampling eliminates the need for a trained phlebotomist to collect samples. It is important to note that for this study serum was the sample type that was used, and it was not possible to examine whether similar results would have been realized had plasma been separated from the venous blood and capillary blood before being subjected to HPLC analysis for retinol determination.

This study adds to a growing body of research that has documented a significant linear correlation between retinol and RBP in serum derived from venous blood samples.[8, 9, 10] Further, the results from this study demonstrated a close correspondence between retinol in venous blood samples and RBP in capillary blood. Moreover, serum RBP, often seen as a surrogate measure for serum retinol, had a significant screening proficiency in classifying VAD among populations. These studies, which have examined the correspondence between retinol and RBP, have suggested that a slightly higher molar cutoff point for RBP should be used when estimating VAD than is employed for retinol. ROC analysis from this study indicated that a cutoff point of RBP < 0.825 µmol/L should be used to estimate VAD prevalence in comparison with the cutoff point of retinol < 0.70 µmol/L. Because many program managers responsible for the implementation of VAD control are most concerned with changes in the prevalence of VAD in response to interventions, this study provides some clear avenues for simplifying specimen collection and lab analysis to support program management.

The study also considered the association between retinol and RBP from venous and capillary specimens controlling for inflammation. Infectious diseases cause an increase in acute-phase proteins such as CRP and AGP. The relative concentration of these two acute-phase proteins indicates the duration of infection, where CRP is raised in the initial hours following infection and reaches its highest levels within two days and normalizes rapidly. In contrast, AGP takes longer to rise and does not reach its highest levels until several days following the infection and remains elevated during convalescence.[19] As has been seen in other studies, there was a strong association between retinol and RBP with inflammation status, particularly in the stage of early convalescence. The fact that the same pattern was not as strong in late convalescence suggests that CRP in this study is a more important parameter in this population in confounding the assessment of VA status.

It has been suggested that one way to handle the depressed parameters of VA status in the presence of inflammation is to exclude those children with inflammation when generating estimates of VAD. The basis for this recommendation is that by only including healthy children in the analysis, low retinol or RBP will reflect overt VAD rather than depressed VA that may be confounded by inflammation. However, this could potentially bias a sample[20] and not provide a true representation of VAD in a population as children with VAD are more likely to be suffering from infection. Others have recommended the use of correction factors to adjust VAD prevalence to account for inflammation in a population,[21]

but these factors have not been refined. Future research is required to better answer these critical questions of data interpretation.

For this study, which aimed to establish the underlying biological relationships between retinol and RBP from different specimen types, capillary blood was collected and stored using microcapillary tubes. It is important to note that capillary tubes still require controlled conditions for sample processing and transportation that may be difficult to attain in the field. One possible alternative may be to use filter paper matrices for storing capillary blood as DBS. DBS methods are available for a growing number of analytes including CRP, [22] thyroid hormones, retinol, and serum transferrin receptor. Several community-based applications have shown this collection method to be a reliable means of simplifying sample storage and transport. Further research will, however, be necessary to examine the most appropriate and field-friendly methods of collecting capillary blood.

The finding of a strong correspondence between retinol and RBP in both venous and capillary samples suggests that investigators have additional options when assessing VA status in community-based studies. The analysis of RBP from capillary blood bears many advantages, including ease in sample collection, accuracy in detection of VAD, affordability, and feasible analysis with standard laboratory equipment. The information derived from this study about the feasibility of using capillary blood for assessing VA status is exciting, yet it is important to emphasize that great measures were taken to ensure that pristine specimens were collected and vigilant efforts were followed to maintain rigor in specimen handling and storage.

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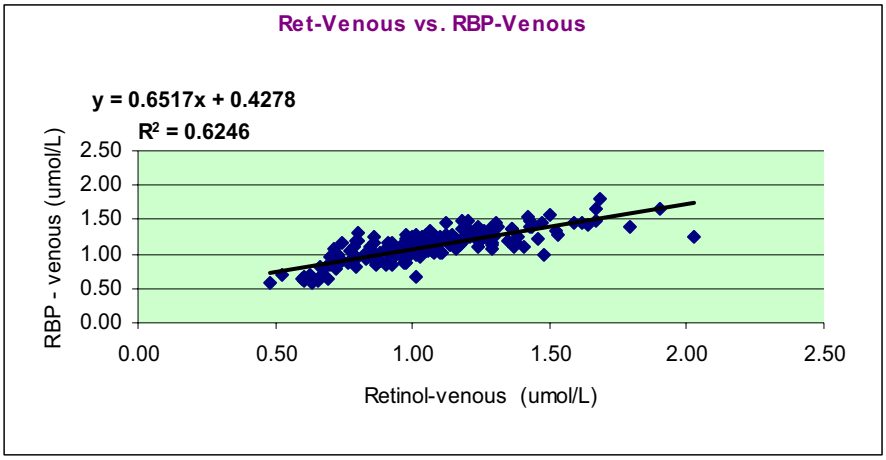
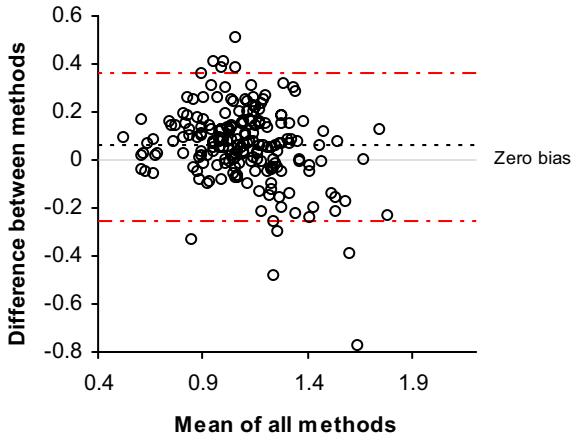
Appendix 1—Correlation Between Parameters of VAD

Retinol-venous vs. RBP-venous

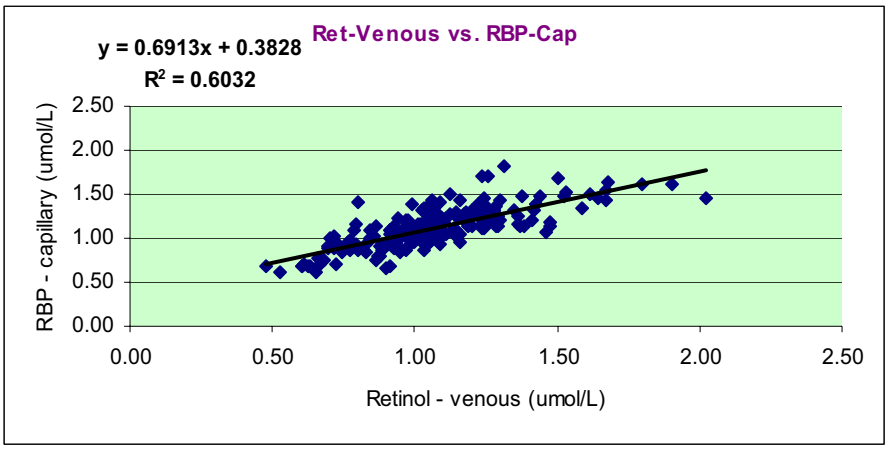


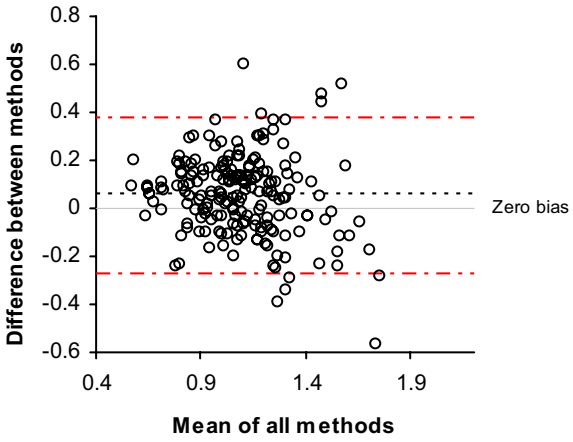


Bias	0.057	
95% CI	0.035	to 0.080

95% Limits of Agreement		**95% CI**	
Lower	-0.255	-0.294	to -0.217
Upper	0.370	0.332	to 0.408

Retinol-venous vs. RBP-capillary

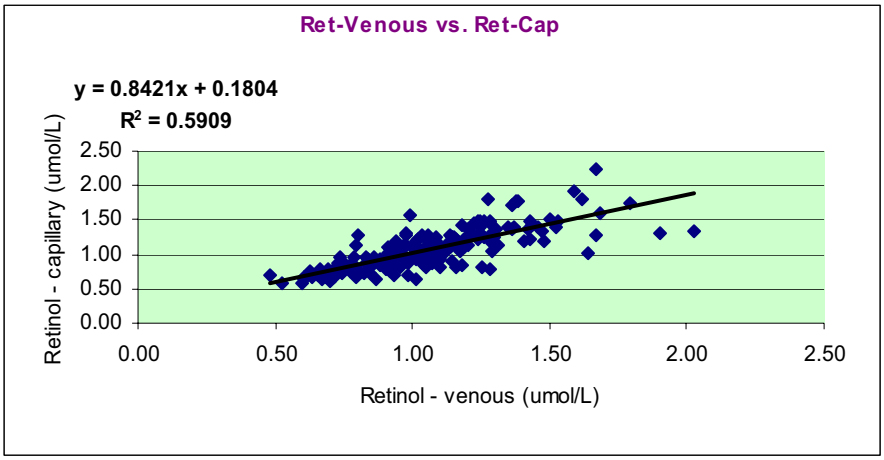


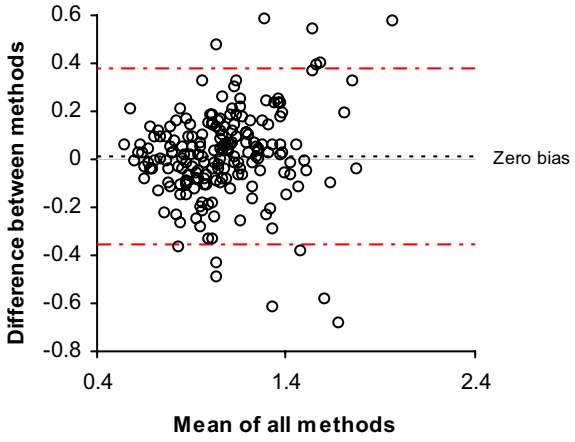


Bias	0.054	
95% CI	0.031	to 0.078

95% Limits of Agreement		**95% CI**	
Lower	-0.273	-0.314	to -0.233
Upper	0.382	0.342	to 0.423

Retinol-venous vs. retinol-capillary





Bias	0.012	
95% CI	-0.014	to 0.039

95% Limits of Agreement		**95% CI**	
Lower	-0.355	-0.400	to -0.310
Upper	0.379	0.335	to 0.424

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