

Total Adipose Retinol Concentrations Are Correlated with Total Liver Retinol Concentrations in Male Mongolian Gerbils, but Only Partially Explained by Chylomicron Deposition Assessed with Total α -Retinol

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ABSTRACT

Background: Liver vitamin A (VA) concentration is the gold standard for VA status, but is not routinely accessible. Adipose tissue VA concentrations, as retinol and retinyl esters, may be correlated to liver VA. α -VA (as α -retinol) is a cleavage product of α -carotene that traces postprandial VA distribution to tissues but cannot recirculate from hepatic stores, providing insight into tissue VA sources.

Objective: We performed a secondary analysis of VA and α -VA in Mongolian gerbil liver and adipose to determine the suitability of adipose tissue VA as a biomarker of VA status.

Methods: Gerbils ($n = 186$) consumed feeds containing 0–15.9 μg (0–55.6 nmol) retinol activity equivalents/g as preformed VA and/or provitamin A carotenoids for 36–62 d in 3 studies. Body fat percentage was determined in the final study by MRI. Serum and liver retinol, α -retinol, and retinyl esters were extracted and analyzed by HPLC or ultra-performance LC (UPLC). Epididymal and retroperitoneal adipose tissue retinol and α -retinol were determined by UPLC after homogenization, saponification, and extraction. Linear regression models with appropriate data transformations identified determinants of adipose VA concentrations.

Results: Liver VA did not predict serum retinol concentrations. After logarithmic transformation of adipose and liver values, liver VA positively predicted both adipose depots' VA concentrations ($P < 0.001$, $R^2 > 0.7$). Adding serum retinol or body fat percentage did not significantly increase the adjusted R^2 . In liver, α -VA concentration explained much of the variation of VA ($P < 0.001$, $R^2 > 0.7$), but far less in epididymal and retroperitoneal adipose ($P = 0.004$ and 0.012 , respectively, $R^2 < 0.4$).

Conclusions: Adipose VA is correlated with liver VA and is a potential biomarker of VA status. It is not fully explained by chylomicron deposition and is negatively affected by serum retinol. Adipose biopsy validation is needed for human applications. *Curr Dev Nutr* 2019;3:nzy096.

Introduction

Vitamin A (VA) is an essential nutrient obtained from preformed retinyl esters in animal products or supplements and from provitamin A carotenoids (e.g., α -carotene, β -carotene, β -cryptoxanthin) in plant sources or supplements. VA is best known for its essentiality in vision as 11-*cis*-retinal, but is also vital for growth, immunity, and cell signaling through the binding of the oxidation product retinoic acid to nuclear receptors (i.e., retinoic acid and retinoid X receptors) (1). Both deficient and elevated concentrations of VA can be harmful (1), so it is necessary to monitor populations at risk of these conditions.



Keywords: biomarkers, adipose tissue, chylomicron remnants, retinol-binding protein, vitamin A

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Abbreviations used: EPI, epididymal; RAE, retinol activity equivalent; RBP4, retinol-binding protein; RP, retroperitoneal; UPLC, ultra-performance LC; VA, vitamin A.

Liver VA concentration is considered the “gold standard” for VA status assessment because >80% of total body VA stores are in hepatic stellate cells in VA-adequate adults (1). Because liver biopsies are not routinely accessible, public health researchers use other biomarkers of VA status to determine the prevalence of VA deficiency, hypervitaminosis, or toxicity (1). Serum retinol is the most commonly used biomarker: the WHO defines a prevalence of >20% of a population with serum retinol concentrations $<0.7 \mu\text{mol/L}$ as representing a serious risk of deficiency (2); however, no concentration has been identified that reflects elevated VA status. Serum retinol suffers from insensitivity (3) and is affected by infection and inflammation (4). Other indicators, such as the modified-relative-dose-response test, qualitatively determine VA deficiency or sufficiency (1). Retinol isotope dilution measures total body VA stores from deficiency through toxicity in groups of individuals using sophisticated mass spectrometric techniques (1). However, an inexpensive biomarker is needed that accurately predicts VA status over a wide range of values that can be broadly applied to allow researchers to determine deficiency and hypervitaminosis A at the population level. Adipose tissue VA concentration, potentially taken from a needle biopsy of subcutaneous fat, could be one such biomarker.

Humans and rodents maintain adipose VA concentrations between 1 and 24 nmol/g tissue (5–8), much lower than liver VA concentrations (100–700 nmol/g in optimal VA status) (1). Estimates of the relative proportion of total VA in adipose to that in liver vary significantly. For example, adipose VA stores were estimated to be 15–20% of those in liver in rats when assuming adipose and liver comprise 15% and 4% of body mass, respectively (9). In Mongolian gerbils (*Meriones unguiculatus*), adipose VA stores are <5% of those in liver using the same assumptions (7). In rats, VA concentrations in liver and several adipose depots varied positively according to VA intake (6, 10). Similarly, an increase in VA concentration occurred in liver and abdominal subcutaneous adipose tissue of ferrets supplemented with β -carotene compared with controls (11). Gerbils fed varying amounts of β -cryptoxanthin that did not have differences in liver VA had no difference in adipose tissue VA (7). These correlations suggest that changes in VA status may be reflected in adipose. Correlation of liver and adipose VA may be due to increased dietary VA delivery to tissues by chylomicra after a meal, rather than resulting from redistribution of retinol secreted from hepatic stores on retinol-binding protein (RBP4) (6). This theory has been supported by studies showing no difference in VA concentrations in extrahepatic tissues (other than the eye) in mice lacking RBP4 compared with wild-type mice (12). In addition, humans with RBP4 mutations do not have overt symptoms of VA deficiency when consuming a VA-adequate diet (13).

α -Retinol is a retinol isomer found in liver and tissues of animals and humans consuming α -carotene (14) that may provide insight into the relative importance of different VA sources without disrupting protein function. Unlike β -carotene, which produces 2 retinal molecules when cleaved, α -carotene yields 1 retinal and 1 α -retinal, which are reduced to their respective alcohol forms, esterified, and packaged into chylomicra. In gerbils fed α -carotene, hepatic α -retinol and retinol were present in equal amounts, indicating that the initial steps of α -retinol metabolism quantitatively trace retinol metabolism (15). However, once in the liver, α -retinol cannot bind RBP4 for secretion into plasma (16), making it an effective tracer of chylomicron distribution, but not hepatic recirculation. VA-sufficient diets deposit adequate VA into tissues

through chylomicra (12), thus not disrupting the hepatic 1:1 ratio of retinol: α -retinol.

The study presented herein was a secondary analysis of 3 studies in gerbils with a range of dietary VA concentrations from deficient through excessive as preformed retinyl esters and provitamin A carotenoids. VA concentrations in liver, epididymal (EPI), and retroperitoneal (RP) adipose were analyzed to evaluate adipose VA concentration as a biomarker of VA status against liver VA concentration. In the third study, body fat was measured to determine the effect of increased fat mass on adipose VA concentration, and α -VA was analyzed to provide insight into the source of adipose VA.

Methods

Animals and feeds

Approval for the 3 studies from which tissues and data were taken was obtained from the University of Wisconsin-Madison College of Agricultural and Life Sciences Animal Care and Use Committee. The primary outcomes and analyses of these studies will be reported elsewhere or have been partially published (17). The feeds and study protocols are summarized in Table 1. Briefly, male Mongolian gerbils ($n = 40$ –80) were fed a VA-deficient feed for 14–21 d, then divided into groups ($n = 5$ –10/group) and given preformed VA and/or provitamin A carotenoids as biofortified (orange) maize and/or orange carrots (Study 1, $n = 80$), dried cassava leaves (Study 2, $n = 40$), or high-carotene orange carrots (Study 3, $n = 66$) for 36–62 d. Study 1 included a low concentration of VA fortificant as retinyl palmitate [0.2–0.4 μg retinol activity equivalents (RAE)/g feed, equivalent to approximately half the estimated daily requirement of gerbils from prior studies (18, 19)]; Study 2 included a retinyl acetate supplement as a positive control; and Study 3 included retinyl palmitate VA fortificant at 1 or 2 times the same estimated daily requirement for gerbils as followed in Study 1 (0.4–0.5 and 1.0 μg RAE/g feed). Control feeds contained white maize and/or white carrots. In Study 3, body fat percentage was determined in live gerbils <30 min before death by whole body MRI (EchoMRI), which was calibrated against a known oil-based phantom. Full matched sets of EPI and RP adipose depots, serum, and liver were not completely available for all gerbils in all 3 studies, therefore sample sizes are noted for each analysis.

Sample collection and preparation

Gerbils were anesthetized by isoflurane and killed by exsanguination through cardiac puncture. Blood, EPI and RP fat pads, and whole liver were collected. Blood was allowed to clot at room temperature for 20 min and then serum was separated by centrifugation at $2800 \times g$ for 15 min at 4°C. All tissues were weighed and stored at -80°C until analysis.

Serum, liver, and adipose tissue VA and α -VA analysis

Serum and liver retinol were determined by HPLC (Studies 1 and 3) and ultra-performance LC (UPLC; Waters Acquity H-class) (Study 2) as previously described (20, 21). In the first 2 studies, total liver VA concentration was determined by summing the area of the retinol peak and all identifiable retinyl ester peaks. In the third study, both total liver VA and α -VA concentrations were determined from a saponified fraction.

TABLE 1 Characteristics of 3 studies combining dietary provitamin A carotenoids and preformed vitamin A in male Mongolian gerbils

	Study 1	Study 2	Study 3
Characteristic			
Number of gerbils	80	40	66
Baseline age of gerbils, wk	4	4.5	4–5
Endline age of gerbils, wk	14.9	12.6	15–16
Vitamin A depletion, d	14	21	14
Treatment time, d	62	36	62
Baseline body weight (mean \pm SD), g	27.9 \pm 2.5	39.4 \pm 2.0	36.7 \pm 3.7
Endline body weight (mean \pm SD), g	74.3 \pm 6.6	63.2 \pm 4.2	71.2 \pm 3.8
Vitamin A components in feed			
Preformed vitamin A	Fortificant in feed ¹	Oil supplement ²	Fortificant in feed ¹
Provitamin A carotenoids	Orange carrot, orange maize	Dried cassava leaves	Orange carrot
Preformed vitamin A intakes, μ g retinol activity equivalents/d ³			
Control group	0	0	0
VA+ group	1.1–2.3	7.1	2.2–2.4
VA++ group	—	—	5.3
Provitamin A intakes, μ g β -carotene equivalents/d ⁴			
Control group	0.1	0	0.1–0.2
Orange carrot, orange maize	111–112	—	—
Orange carrot, white maize	72.3–79.4	—	116–127
White carrot, orange maize	33.4–39.5	—	—
Dried cassava leaves	—	6.6–6.7	—

¹ Provided as retinyl palmitate [“Dry Vitamin A Palmitate” (250,000 IU VA/g; DSM Nutritional Products Ltd.)].

² Provided as retinyl acetate (Sigma-Aldrich).

³ 1 μ g retinol activity equivalent = 1.15 μ g retinyl acetate or 1.83 μ g retinyl palmitate.

⁴ 1 μ g β -carotene equivalent = 1 μ g β -carotene or 2 μ g β -cryptoxanthin or α -carotene.

For adipose VA concentrations in all 3 studies, \sim 200 mg of tissue was weighed into a 13 \times 100 mm glass culture tube (Fisher Scientific); 100 μ L 8'- β -apo-carotenal (\sim 15 ng, as internal standard; Sigma-Aldrich), 2 mL dichloromethane, and 1 mL methanol were added for a modified Folch total lipid extraction (22, 23). The tissue was mechanically homogenized (T10 ULTRA-TURRAX, IKA), mixed with a vortex for 30 s, and centrifuged at 1400 \times g at room temperature for 10 min to remove solids. The organic layer was washed by the addition of 0.9% NaCl_(aq), mixed for 30 s, and centrifuged as above for 5 min. Also at room temperature, the extracted lipid was dried under N₂ and saponified by resuspension in 1 mL 5% ethanolic KOH (wt:vol) and 0.1% butylated hydroxytoluene (wt:vol) for 10 min at 60°C (24). One milliliter of distilled H₂O was added to quench the reaction and then the solution was extracted twice with 3 mL hexanes. The pooled organic layers were dried under N₂ and resuspended in 100 μ L 75:25 methanol:dichloroethane (vol:vol). Two microliters were injected onto the UPLC equipped with a guard column, a C18 1.8- μ m, 2.1 \times 150 mm column (Waters Acquity HSS), and a heater set to 30°C (Waters). Solvent A was 70:25:5 acetonitrile:water:isopropanol (vol:vol:vol) and solvent B was 75:25 methanol:isopropanol (vol:vol). Each sample was run for 29 min according to a previously described gradient (25). Retinol and α -retinol were quantified at 325 and 311 nm, respectively, by external standardization to HPLC-purified, UV-visible spectrophotometer-authenticated standards [saponified retinyl acetate (Sigma-Aldrich) and α -retinyl acetate synthesized using α -ionone in

a published procedure for retinyl acetate (26)], after correction for recovery of internal standard.

Statistical analysis

Preliminary analysis of the data was performed using Spearman's rank-sum coefficient, after which single and multiple linear regressions were performed using the “LM” procedure in the R statistical package (27). Two extreme outliers for EPI adipose VA concentration (1 in each of Studies 2 and 3, Figure 1A) were identified by Grubb's test for outliers ($P < 0.05$ within each study) and those animals were retroactively excluded from all statistical analyses. These values had excessive influence on regression coefficients [e.g., Cook's distance $>$ (4 \times mean Cook's distance) in the full model described in Supplemental Table 1], resulting in conclusions of strong significance that became non-significant when those points were removed. Liver and adipose VA concentration were transformed by base-10 logarithm (\log_{10}) to achieve normality of errors and homoscedasticity in the general linear model.

Two separate ANCOVAs were performed to explain EPI or RP adipose VA concentration using both independent variables available for all 3 studies (liver VA and serum retinol concentration) as well as the study (1, 2, or 3) as a covariate. For each analysis, we compared a model using different slopes for each study with a model using the same slope for each study. In both the EPI and RP analyses, the different-slopes model did not explain more variance than the simpler same-slopes model according to the additional sum of squares test.

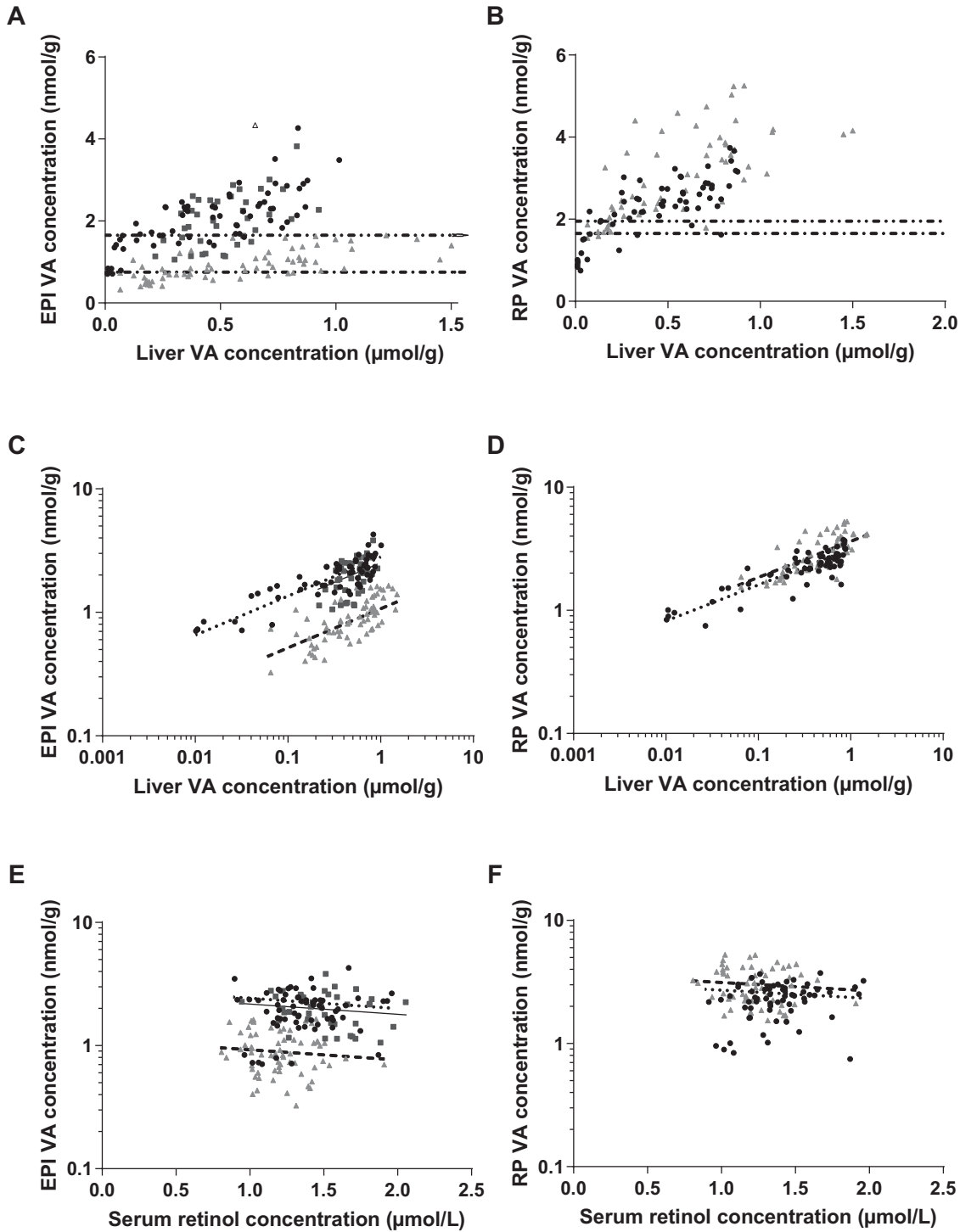


FIGURE 1 Tissue VA (as retinol + retinyl esters), determined by HPLC (liver and serum) or UPLC (adipose tissue) in Studies 1 (black circles), 2 (dark grey squares), and 3 (light grey triangles). RP tissue was only available in Studies 1 and 3. (A, B) (— · —) Example cutoff for deficiency for Studies 1 and 2. (— · —) Example cutoff for deficiency for Study 3. (A) Liver and EPI retinol concentrations. The data point with an open symbol was an outlier and excluded from further analysis. A further outlier at (0.33, 6.50) was off-axis and also excluded. (B) Liver and RP retinol concentrations. (C–F) ANCOVA including liver, serum, and either EPI (C, E) or RP (D, F) tissue VA was performed after base-10 logarithmic transformation of liver and adipose VA concentrations in Studies 1 (.....), 2 (—), and 3 (— · —), with study as a covariate. (C, D) Liver VA concentration explained 28% (D, EPI) and 57% (E, RP) of the variation in adipose VA concentration ($P < 0.001$). (E, F) Serum retinol concentration explained a negligible fraction of the variation in adipose VA concentration. EPI, epididymal adipose; RP, retroperitoneal adipose; UPLC, ultra-performance LC; VA, vitamin A.

Models for EPI and RP were constructed in Study 3 to determine if any variability of EPI and RP adipose VA concentrations was due to body fat percentage. The model included \log_{10} -liver VA and serum retinol as independent variables and tested the significance of inclusion or exclusion of body fat percentage using the aforementioned additional sum of squares test.

ANCOVA was used to determine if EPI and RP adipose VA were correlated within studies, and if that correlation varied between studies, using RP adipose VA concentration as the dependent variable and EPI adipose VA concentration as the independent variable, including study as a covariate. In this case, both the slopes were clearly different, so no further reduction of the model was applied. VA and α -VA concentrations in liver and both adipose tissues were analyzed using ANCOVA to explain VA concentrations from all sources, with α -VA from dietary deposition as an independent variable and treatment group as a covariate. The covariate was necessary because each of the 3 groups had different fortificant concentrations (i.e., 0, 0.5, or 1 μg RAE/g feed) and therefore different ratios of sources of VA to α -VA available in their feed. As above, the different-slopes and same-slopes models were compared and the same-slopes model was used. The regression in liver was repeated with and without including the gerbils that were missing RP tissues to be comparable with the EPI and RP regressions, respectively.

Data are reported as mean \pm SD. Significance was defined as $P \leq 0.01$ rather than $P \leq 0.05$ to account for multiple testing throughout analyses; however, the importance of an independent variable was primarily examined by the magnitude of its contribution to variation in the dependent variable (measured as adjusted R^2); P values for these changes were determined from the additional sum of squares test for completeness. To plot multiple regression lines, the independent variable that was not plotted was held constant at its mean effect. Sensitivity and specificity for a biomarker were calculated as the percentage of VA-deficient gerbils correctly identified as such and the percentage of VA-sufficient gerbils correctly identified as such, respectively. Example cutoffs for population health studies were determined by calculating sensitivity and specificity for cutoff values every 0.05 nmol/g from 0 to 5, then maximizing Youden's index ($J = \text{sensitivity} + \text{specificity} - 1$) (28).

Results

Serum retinol is not correlated with liver VA

Liver VA and serum retinol concentrations were not correlated by Spearman's rank-sum test in any study (Supplemental Figure 1). No gerbil had a serum retinol concentration <0.7 $\mu\text{mol/L}$, the current WHO-recommended cutoff to define VA deficiency in humans (2), despite 12 gerbils with liver stores defined as VA deficient (<0.1 $\mu\text{mol/g}$) (1).

Adipose VA concentration correlates with liver VA concentrations

Study 1, which spanned extremely deficient to high-normal liver VA stores (Figure 1A, B), revealed that a logarithmic data transformation was appropriate for both liver and adipose VA concentrations. The final

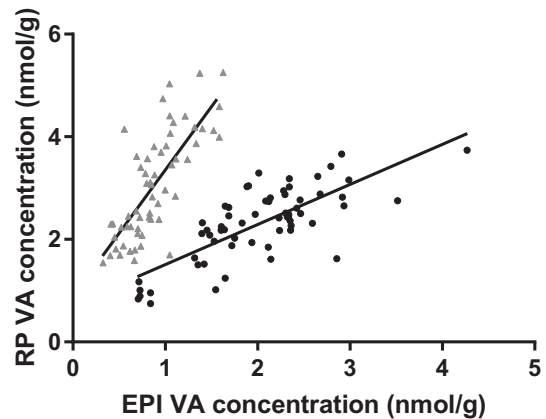


FIGURE 2 EPI and RP VA concentrations are correlated in gerbils. Adipose retinol concentrations were determined by UPLC and plotted against each other for Studies 1 (black circles) and 3 (light grey triangles). ANCOVA with study as a covariate revealed that retinol concentrations were not the same between depots, the relations were different between studies, and they were significantly related overall (adjusted $R^2 = 0.64$, $P < 0.001$). EPI, epididymal adipose; RP, retroperitoneal adipose; UPLC, ultra-performance LC; VA, vitamin A.

models explaining EPI and RP adipose VA concentration were both significant ($P < 0.001$, Supplemental Table 1, Figure 1C–F). The slopes of the regressions were not different between any study and intercepts did not significantly vary between Studies 1 and 2 in EPI adipose depots; Study 3 had a significantly lower intercept than Studies 1 and 2 (EPI) and a higher intercept than Study 1 (RP) ($P < 0.001$). In EPI and RP adipose, \log_{10} -transformed liver VA concentration, serum retinol concentration, and study accounted for 78% and 70% of total variance (measured as adjusted R^2), respectively, 28% and 57% of which was due to liver VA concentration, respectively. Conversely, removing serum from the model did not change the adjusted R^2 (<0.01 difference in either model, $P = 0.02$ and 0.08 , respectively). EPI and RP adipose VA concentrations were correlated (adjusted $R^2 = 0.64$, $P < 0.001$; Figure 2, Supplemental Table 2); however, the relation was different between studies ($P < 0.001$).

Adipose VA concentrations were evaluated as a potential qualitative biomarker of deficient liver VA stores (i.e., <0.1 $\mu\text{mol/g}$) by calculating example cutoff values to predict deficiency or sufficiency (Figure 1A, B). In Studies 1 and 2, a reference value of 1.65 nmol VA/g adipose tissue maximized Youden's index in both EPI ($J = 0.76$) and RP ($J = 0.85$), resulting in a sensitivity of 100% (12 of 12 gerbils) in EPI and 91% (10 of 11 gerbils) in RP, and a specificity of 76% (70 of 92 gerbils) in EPI and 94% (48 of 51 gerbils) in RP. Study 3 was examined separately because the gerbils had significantly different adipose VA concentrations over the range of liver values, as revealed by the different intercept coefficients in the ANCOVA models (Supplemental Table 1). Only 2 gerbils in Study 3 had deficient liver VA stores, and a reference value of 0.75 nmol/g EPI or 1.95 nmol/g RP adipose yielded 100% sensitivity (2 of 2 gerbils) and a specificity of 63% (40 of 52 gerbils) in EPI or 86% (49 of 52 gerbils) in RP to predict values <0.1 $\mu\text{mol/g}$ liver.

TABLE 2 Distribution of vitamin A in male Mongolian gerbils in Study 3, which included carrots as a provitamin A source and retinyl palmitate as a fortificant¹

	Liver	Adipose	Serum	Total body
Total vitamin A concentration ²	0.585 ± 0.353	0.975 ± 0.544	1.21 ± 0.21	—
Mass/volume of tissue ³	2.61 ± 0.565	8.13 ± 3.29	1.72 ± 0.13 ⁴	55.9 ± 4.1
Total vitamin A in tissue, μmol	1.55 ± 1.09	0.0077 ± 0.0052	0.0021 ± 0.0004	1.56 ± 1.10 ⁵
Total body vitamin A stores, %	99.4	0.5	0.1	100

¹Values are means ± SDs, $n = 66$.

²Values are in $\mu\text{mol/g}$ (for liver), nmol/g (for adipose), and $\mu\text{mol/L}$ (for serum).

³Values are in g except for serum (mL).

⁴Calculated assuming 7.7 mL blood/100 g body weight (29) and a serum fraction of blood of ~ 0.4 .

⁵Calculated as the sum of total vitamin A in these 3 tissues, which are predicted to contain >90% of all vitamin A in the body (1).

Body fat percentage does not predict adipose VA concentrations

In Study 3, the body fat percentage ($12\% \pm 4.3\%$) determined by MRI ranged from 3% to 24%. The estimated contribution of adipose retinol to total body VA stores, which was determined by multiplying the VA concentration by the MRI-predicted total fat mass, was minimal (Table 2). The addition of body fat percentage to the models predicting adipose VA (in Study 3 alone, assuming the inclusion of \log_{10} -liver VA and serum retinol concentration; Supplemental Table 3) in EPI and RP adipose did not affect the model, resulting in negligible changes in adjusted R^2 from 49% to 51% ($P = 0.047$) and 58% to 60% ($P = 0.061$) upon inclusion, respectively.

α -Retinol concentrations

In Study 1, adipose α -VA concentrations ranged from 0 to 0.77 nmol/g tissue, but liver α -VA concentrations were not measured in that study. α -VA concentrations were not quantifiable in Study 2 because carrot was not fed (Table 1). In Study 3, VA concentrations were more strongly correlated to α -VA concentrations in liver than in adipose (Supplemental Tables 4 and 5): variation in liver α -VA (including or excluding samples missing from the RP set) accounted for 62% or 72% of the variation in retinol, respectively ($P < 0.001$; Figure 3A), whereas α -VA only accounted for 16% and 25% of the variation in EPI and RP adipose VA concentrations, respectively ($P < 0.001$, Figure 3B, C).

Discussion

Liver and adipose VA concentrations maintained a nonlinear correlation in all 3 studies in 2 different adipose depots, hence adipose VA concentrations represented a potential biomarker of liver VA and therefore VA status. Body fat percentage and serum retinol concentrations were not significant predictors of adipose VA concentration. Putative reference ranges for VA deficiency using adipose concentrations gave 63–94% specificity and 86–100% sensitivity to diagnose VA deficiency in the studies from which they were generated, which was not possible with serum retinol concentrations. The adipose depots examined were collected owing to their size and ease of collection in gerbils. Concentrations varied between depots and between studies, with RP having ~ 4 times the VA concentration of EPI in Study 3, but

similar concentrations were observed in both depots in Study 1. In rats, adipose VA was similarly correlated with VA status, but was not different between depots (6). For translational applications in humans, targeted studies comparing subcutaneous fat concentrations with isotope dilution-estimated or biopsy-determined liver VA status in more advanced models (e.g., rhesus monkeys, swine) or humans should be conducted, because of the relative complexity of the human diet and tissues compared with gerbils consuming an unchanging diet with few (≤ 3) VA sources. Furthermore, the presence of 2 outliers for EPI adipose VA concentration indicates the potential for this relation to be disrupted among individuals. For this reason, adipose VA concentration may be a technique more useful in population surveys than for individual VA status assessment; in the former, the influence of rare outliers would be minimized.

Interestingly, the slope of the liver-adipose VA regression lines did not vary between studies. The logarithmic relation indicates that there is a slowing of VA storage in adipose tissue as liver VA increases, perhaps indicating a maximum capacity or nonlinear induction of degradation. As a potential biomarker of VA status, especially for excessive VA stores, this is an important consideration.

Study 3's intercept differed from those of the first 2 studies; adipose retinol was lower than the other 2 studies in EPI and higher than Study 1 in RP. This difference could be attributable to heterogeneity between gerbils in the first 2 studies and those in the third. To our knowledge, the only impactful difference was that Study 3 gerbils were older and more variable in age than those in the first 2 studies. Age influences VA-modulating parameters such as body weight and immune function (1), and the impact of age on adipose VA metabolism could be an interesting avenue of research given the information already known on the interaction of adipose retinoid metabolism and disruptions in whole body energy metabolism, including insulin resistance and hepatic steatosis (30, 31). Unfortunately, individual gerbil age was not available in Study 3 because gerbils were shipped together and therefore age could not be controlled for as a predictor of stores. Other differences between studies could be due to a longer time at the supplier where perhaps they were consuming a different feed or being exposed to various stressors that uniformly modulated their adipose stores, somehow differently in either depot. The studies were also completed at different times, which could mean that some genetic event or crossbreeding had occurred at the supplier between studies, as the status of the gerbil genome

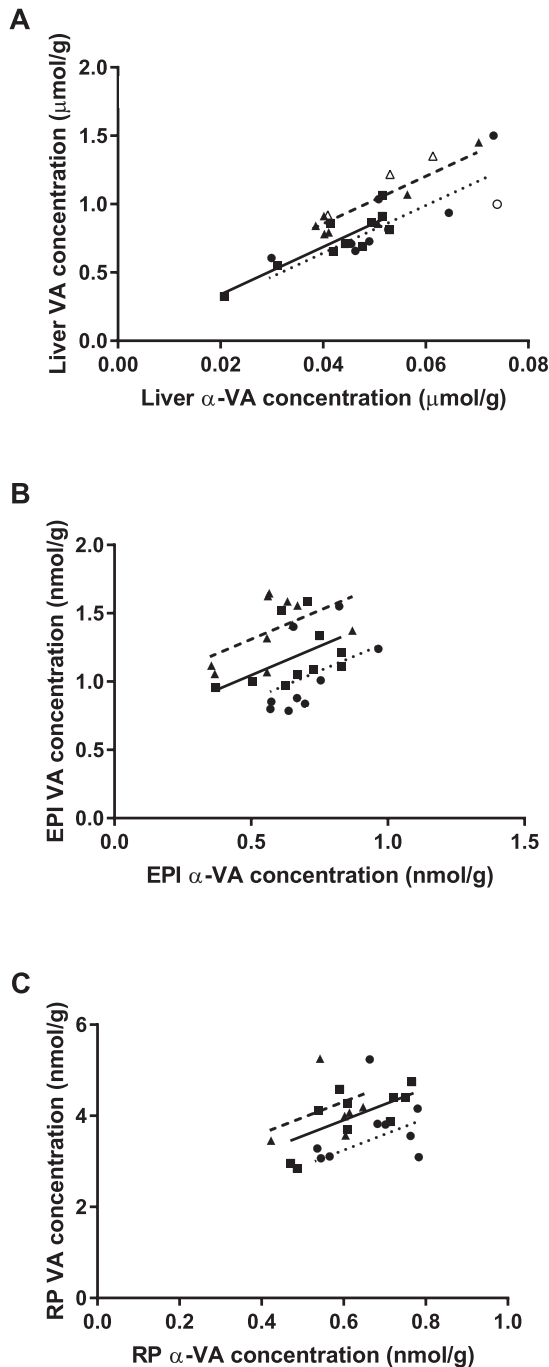


FIGURE 3 The fraction of the variation in tissue VA concentration from all sources explained by chylomicron deposition in ANCOVA, measured as α -VA concentration, both determined by HPLC (liver) or UPLC (adipose). Dietary treatment was added as a covariate to control for different quantities of preformed VA [0 (\bullet ,); 0.5 (\blacksquare , ---); 1 (\blacktriangle , ---) $\mu\text{g/g}$ feed] in each group. (A) α -VA concentrations explained $>60\%$ ($P < 0.001$) of the variation in VA concentration in liver. Data points with open symbols were from gerbils which were included in the EPI analysis but lacked RP samples. (B, C) α -VA concentration explained 15% ($P = 0.012$; B, epididymal) and 25% ($P = 0.004$; C, retroperitoneal) of the variation in retinol concentration in adipose. EPI, epididymal adipose; RP, retroperitoneal adipose; UPLC, ultra-performance LC; VA, vitamin A.

is less well-monitored than for other rodent species (e.g., C57BL/6J mice). Regardless of the source, this difference between studies indicates that the use of adipose as a biomarker likely requires standardization of reference ranges to each population or age group for application; however, regardless of differences between studies or adipose depots, the correlation of adipose and liver VA concentrations was maintained.

Blaner et al. (6) proposed that the changes in adipose and liver VA concentrations without corresponding changes in serum retinol indicated that dietary deposition was responsible for tissue concentrations because this variation was noted when varying the dietary amount of VA. Since then, studies from the same group have shown that chylomicron delivery was sufficient to maintain mean VA concentrations in tissues other than the eye in mice lacking RBP4 (12) or its transmembrane receptor Stimulated by Retinoic Acid 6 (STRA6) (32). These data suggest that RBP4 delivery of VA to tissues other than the eye in dietary VA sufficiency is dispensable. In the absence of a method to differentiate dietary and recirculated hepatic VA within individual animals, these conclusions are also supported by our study, given that the differing liver values between groups of gerbils were achieved by variations in dietary VA.

Upon analyzing α -VA in α -carotene-consuming gerbils in Study 3, however, chylomicron-delivered α -VA accounted for variation in liver VA stores more strongly than in adipose VA stores. α -VA and VA are initially derived from the diet at some ratio, which could be measured in chylomicra in a targeted study. This ratio varies according to the amount of additional VA fortificant added to the feeds of 3 different groups (0, 1, and 2 μg RAE/g feed), so this concentration was used as a covariate in analysis of ratios of VA to α -VA in tissues. Although this ratio could theoretically change by discrimination for or against α -VA by physical phenomena (e.g., packing into lipoproteins) or enzyme rates (e.g., differential esterification or hydrolysis), evidence suggests that it is delivered to the liver at the expected ratio (15). In this case, we propose that this discrimination is due to RBP4 delivery of retinol (but not α -retinol, which cannot bind RBP4) to the tissue of interest according to VA status. Less likely, but still possible, is that α -VA could be added by differential cleavage of α -carotene and the generation of α -retinol by adipose tissue β -carotene-15, 15'-oxygenase in situ; however, no α -carotene was detected by photo-diode array analysis in the adipose tissue at 450 nm during UPLC analysis.

Liver VA stores contain $>99\%$ of the VA in the tissues examined in these gerbils (Table 2); therefore, it is not surprising that the feed (rather than some other storage organ) is the major source of VA in the liver, which is apparent from dietary-derived α -VA explaining $>60\%$ of the variation in liver VA. Conversely, although a ratio of α -VA to VA is deposited in both adipose depots examined, the VA concentration appears to be changed by a variable amount in each animal, weakening the correlation such that α -VA explains $<25\%$ of the VA concentrations in adipose tissue. This could suggest that RBP4 delivery of hepatic VA stores to peripheral tissues is important to maintain a particular adipose VA concentration.

In our 3 studies and other reports (1), serum retinol concentration did not positively correlate with dietary intake or liver VA and did not predict adipose VA. The mechanism behind the relation between liver and adipose VA concentrations without strongly correlated changes in serum retinol concentrations could lie in a change in flux of VA out of liver to serum and then into tissue despite a long-term,

near-constant concentration in serum. This flux may be controlled by another metabolite (e.g., retinoic acid, retinyl- or retinoyl- β -glucuronide adducts) affecting either or both of liver release and adipose uptake, or by secreting liver VA in short pulses that acutely increase adipose VA uptake but are not reflected in fasting serum retinol measurements made at the time of death. Retinoyl- β -glucuronide decreased serum retinol concentrations in rats after oral administration, demonstrating potential regulatory action (33). Further research must explore these potential effects to determine how the correlation between adipose and liver VA is maintained.

Biomarkers of VA status that are minimally invasive are necessary for population assessment (1), especially with the widespread use of VA supplements and fortificants. Although stable-isotope methods are an indirect method to assess VA status in research settings, they are cost-prohibitive and technically challenging to perform routinely on large groups of subjects for population monitoring. Thus, a method to predict VA status during a single visit, rather than multiple blood draws over several weeks and sophisticated isotope MS, would improve the ability of health agencies to assess the impact of VA interventions.

This study showed that adipose VA concentrations were correlated to liver VA concentrations in gerbils, but there was a potential plateau at higher concentrations. This plateau, as well as clinically relevant concentrations corresponding to deficiency, likely vary according to factors such as species, dietary VA source, age, and genetic background. For example, human adipose VA concentrations have been reported to be similar to, or ≤ 3 times higher than, adipose VA concentrations in these gerbils (8, 34, 35), but still lower than those in rats (9). Within the results reported here, studies with different ages and feeds yielded different mean adipose VA concentrations despite following the same logarithmic correlation. Finally, we recognize that historical measurements of human adipose and liver VA concentrations have not yielded a clear correlation between the 2 (5, 36); however, these evaluations preceded modern chromatographic techniques, such as HPLC or UPLC, likely lacking the accuracy to detect small changes within a narrow range of adipose VA concentrations. Thus, human studies using sensitive analytical techniques are needed to determine the correlation of adipose and liver VA concentrations within and between populations to determine the utility of adipose retinol concentration as a biomarker of VA status.

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