Original Research

Modified relative dose response values differ between lactating women in the United States and Indonesia

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Impact statement

Vitamin A (VA) deficiency is a major health issue globally, and lactating women are particularly vulnerable due to increased needs for milk production. Accurate detection of VA deficiency is important; however, most population surveys measure VA status using serum retinol, which is affected by inflammation and lacks sensitivity. The modified relative dose response (MRDR) test qualitatively distinguishes between VA deficiency and sufficiency and could improve population surveys if completed in a randomly selected subsample of individuals in surveys. The original relative dose response test required two blood samples, while MRDR requires only one, a significant improvement in accessibility of the technique by decreasing burden on subjects and investigators. This work demonstrates significant deficiency in Indonesian women compared with US women. In combination with previous research using lactating sows, these human data support milk as a surrogate for blood in the MRDR, which may be less invasive, but requires further validation.

Abstract

The modified relative dose response (MRDR) test distinguishes between vitamin A (VA) deficiency and sufficiency using the molar ratio of 3,4-didehydroretinol to retinol (DR:R) in serum 4–6 h after administering 3,4-didehydroretinyl acetate (DRA). Research in sows demonstrated that serum and milk DR:R are correlated. Two studies determined VA status in lactating women and investigated breast milk as a surrogate for serum in the MRDR test in VA-adequate women. A secondary outcome compared serum with milk carotenoids in US women. Lactating US (24–40 years old, $n = 25$) and Indonesian (22–40 years old, $n = 18$) women were given 8.8 μmol DRA. Subjects were allocated to three collections (3–48 h post-dose) for blood and milk ($n = 4$–10/time point). DR, retinol, and carotenoids were determined by HPLC. Serum DR:R time-courses were evaluated in all women and DR kinetics analyzed by noncompartmental modeling in the US and VA-deficient Indonesian women. Indonesian women had a higher proportion of VA deficiency by MRDR (61%) than US women (0%). Milk DR concentration was higher than serum. In US women, serum and milk DR:R were correlated at 5 h ($r = 0.86$, $P < 0.01$) but not at 6 or 7 h. Serum DR kinetics ($t_{max} = 8$, $t_{1/2} = 15.3$ h) were similar to those in VA-adequate lactating sows ($t_{max} = 7.5 \pm 1.9$, $t_{1/2} = 17.4 \pm 4.5$ h) but varied in milk (human: $t_{max} = 12$, $t_{1/2} = 22.4$ h; sow: $t_{max} = 14.4 \pm 9.8$, $t_{1/2} = 71.8 \pm 51.2$ h). Carotenoids in milk differed from serum ($P < 0.001$), with β-cryptoxanthin enrichment. Serum but not milk VA kinetics were similar between women and sows. Milk DR:R may represent a simpler VA biomarker in lactating women vulnerable to VA deficiency, but requires further validation.

Keywords: β-cryptoxanthin, breast milk, didehydroretinol, humans, Indonesia, lactation, vitamin A

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Introduction

Vitamin A (VA) is an essential nutrient and VA deficiency is of public health concern in low- and middle-income countries.[1] The gold standard to determine VA status is liver biopsy[2]; however, access to this tissue is limited except under special circumstances. A recent study in adult US cadavers revealed a prevalence of 22% VA deficiency measuring liver samples collected at autopsy.[3] Currently, severe VA deficiency in a population is defined by the World Health Organization (WHO) as >20% with serum retinol concentrations <0.7 μmol/L in combination with other risk factors.[4] Serum retinol, however, is homeostatically regulated, and is not depressed except during extreme deficiency, and is affected by inflammation, leading to poor sensitivity.[5]

The modified relative dose response (MRDR) test is an adaptation of the relative dose response (RDR) test, which relies on the hepatic accumulation of *apo*-retinol-binding

protein (RBP4) during VA deficiency.[2,6] In the RDR, subjects consume a physiological dose of retinyl acetate or palmitate, and the serum concentration of retinol at 5 h post-dose is compared with that at baseline using the formula $(A_5 - A_0)/A_5$;[6] a value >0.2 (20%) indicates significant hepatic accumulation of RBP4 due to VA deficiency, which was confirmed by liver biopsy.[7,8] The MRDR test eliminates the need for the baseline blood draw by using 3,4-didehydroretinol (DR; commonly known as vitamin A_2), which is typically not present in the human diet to a large extent, except in some fish livers.[9] Therefore, the baseline value can be assumed to be negligible. The MRDR value is the molar ratio of DR to retinol (DR:R) measured 4 to 6 h after administration of DR; MRDR values ≥0.060 indicate deficiency.[2]

Blood draws represent a significant deterrent to population VA status assessment due to cultural stigma, fear of needles, and mistrust of investigators.[10] Any method to avoid drawing blood, such as the change from two blood draws in the RDR to one in the MRDR or using capillary blood instead of venipuncture, is desirable to increase willingness to participate and decrease dropout rates. We have previously shown that the MRDR value in milk is correlated to that in serum in VA-adequate and VA-depleted lactating sows.[11,12]

We propose that breast milk DR:R could serve as a surrogate marker for serum DR:R in the MRDR test in lactating women. We investigated this hypothesis by administering the standard adult dose of 8.8 μmol 3,4-didehydroretinyl acetate (DRA) to lactating women residing in Midwestern US and following serum and milk DR:R for 48 h. The correlation was determined at specific time points and DR kinetics were analyzed by noncompartmental modeling and compared with previous studies in sows.[11,12] Major carotenoids in the serum and milk were analyzed by HPLC and compared between the two pools. A similar study design was used in lactating Indonesian women due to a high prevalence of deficiency in prior studies to compare with the US women.[13–15]

Methods

Subjects and study design

The Health Sciences Institutional Review Board of the University of Wisconsin-Madison approved the studies, and the Department of Health Ethical Committee of Indonesia concurrently approved the parallel Indonesian study. US resident ($n = 25$) and Indonesian lactating mothers ($n = 18$) ≥22 y of age who had been lactating for at least eight weeks and planned to continue breastfeeding during the study period were recruited for participation. Subject baseline characteristics were collected (Table 1). US subjects completed a Harvard Food Frequency Questionnaire (FFQ) upon enrollment to determine typical dietary intake patterns of VA and carotenoids.[16] The Harvard School of Public Health quantified the FFQs, and the results were not shared with the participants. Subjects were randomized into one of five blood-draw timing schedules and told to avoid consuming supplements for four days prior to and during the study. Subjects received 8.8 μmol DRA in corn oil followed by a tablespoon of peanut butter on a bagel (US) or fried noodles and white potatoes (Indonesia) to aid absorption. Subjects underwent three blood and milk sample collections at 3, 4, 5, 6, 7, 8, 10, 12, 14, 24, or 48 h. Infants of Indonesian women dosed with DRA had a single blood collection at 24 or 48 h (Figure 1). Subjects could leave the facility or remain on-site during their breaks; low-VA meals and snacks were provided to avoid interference with the test. The primary outcomes were the correlation of milk and serum DR:R at different times and DR kinetics in the US women and to compare serum DR:R between the US and Indonesian women. The secondary outcome compared serum with breast milk carotenoid concentrations in the US cohort.

Table 1. Anthropometric measures and vitamin A biomarkers in US and Indonesian women.[a]

Measurements	US Women ($n = 25$)	Indonesian women ($n = 18$)
Age, y	30.2 ± 3.7	28.6 ± 5.7
BMI, kg/m^2	25.4 ± 4.0	21.8 ± 1.7
Lactation duration[b], d	92.4 ± 25.5	97.9 ± 15.3
Serum retinol concentration, μmol/L	1.78 ± 0.36[c]	0.80 ± 0.40[c]
Breast milk retinol concentration, μmol/L	1.56 ± 0.96[c]	0.85 ± 0.50[d]

[a]Values are mean ± SD.
[b]Lactation duration is equivalent to infant's age.
[c]Mean of all subjects at all timepoints because these values did not vary over time.
[d]Mean of all available values in these women.

Sample collection

Subjects were instructed to collect 20 mL breast milk from one breast approximately 5 min after infant suckling or pumping had been initiated to avoid foremilk. Blood samples were collected into 5 mL Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) or by manual syringe for the infants and subsequently transferred to Vacutainers. Samples were allowed to clot at room temperature and serum was separated by centrifugation. All samples were stored at −70°C until analysis in the US or −30°C in Indonesia.

Serum and breast milk 3,4-didehydroretinol to retinol ratio analysis

US serum and breast milk samples were analyzed for retinol and the MRDR value with standardized HPLC methods.[17] Indonesian samples were analyzed for retinol and DR in Indonesia using the same methods. Serum (400 μL) was treated with 500 μL ethanol and extracted twice with hexanes; retinyl acetate served as an internal standard. Milk (1 mL) was treated with 1.5 mL ethanol with 0.1% (wt:vol) butylated hydroxytoluene as an antioxidant, saponified with 400 μL 50:50 (wt:vol) potassium hydroxide in water at 45°C for 60 min, and extracted with hexanes three times; C23-β-*apo*-carotenol was used as an internal standard.[18] Pooled hexane extracts for serum and milk

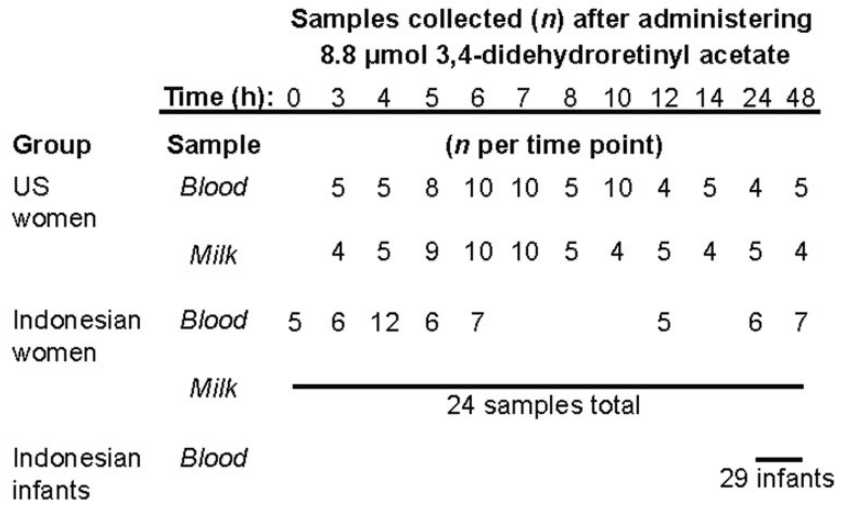

Figure 1. Each US and Indonesian woman underwent a total of three blood draws and breast milk collections at 3, 4, 5, 6, 7, 8, 10, 12, 14, 24, or 48 h; Indonesian infants underwent a blood draw at 24 or 48 h. A higher sample size was allocated to time points 5, 6, and 7 h, which is when MRDR values are typically assessed in serum. MRDR, modified relative dose response.

were dried under argon and reconstituted in 50 µL and 100 µL 75:25 methanol:dichloroethane (vol:vol), respectively, for HPLC analysis; 35 µL or 25 µL serum or milk reconstituted extract, respectively, was injected onto an HPLC system equipped with a Resolve C18 column (3.9 × 150 mm, 5 µm, Waters, Milford, MA) with an isocratic mobile phase of 89:11 methanol:water (vol:vol, with 0.73 g/L triethylamine) at a flow rate of 1 mL/min. Retinol and DR concentrations were quantified at 350 nm using standard curves of each analyte. The definition of VA deficiency using the MRDR test was considered a serum molar ratio of DR:R ≥0.060 4–6 h post-dose.[2]

Analysis of serum and breast milk carotenoids

Serum or milk (0.5 mL) was treated with 1 mL ethanol (0.1% butylated hydroxytoluene, wt:vol) and extracted three times with 1 mL hexanes. Pooled hexane layers were dried under argon and resuspended in 100 µL 50:50 methanol:dichloroethane (vol:vol); 50 µL was injected onto an HPLC system equipped with a Sunfire C18 column (4.6 × 250 mm, 5 µm; Waters, Milford, MA) with a published gradient running at 2 mL/min.[19] Peaks were identified by retention time and absorption spectra from photodiode array detection and concentration were quantified using external calibration with purified standards for each carotenoid. β-*apo*-8′-carotenal was used as an internal standard.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software, Inc.; San Diego, CA). Retinol concentrations over time (including deficiency as a covariate in Indonesian women) were analyzed by ANOVA; retinol concentrations in serum and milk were compared by paired Student's *t* test. Carotenoid profiles of serum and breast milk were compared by χ^2 test; total serum and milk carotenoid concentrations were compared by paired Student's *t* test. Correlation coefficients and regression equations with 95% confidence intervals for milk and serum DR:R at different time points were determined by simple linear regression. Noncompartmental analysis of DR kinetics was performed using PKSolver, a VBA (Visual Basic)-based add-in for Microsoft Excel[20] by taking the mean of all measurements at each time point and performing the modeling as a pooled "individual" analysis due to the number of time points for each subject. Loss of the DR tracer from serum was assumed to occur according to first-order kinetics.[21] Significance in all cases was considered at $P \leq 0.05$.

Table 2. Daily dietary intake of US lactating women as assessed by the Harvard Food Frequency Questionnaire.[a]

Nutrient	Daily intake
Calories, kcal	2240 ± 497
Carbohydrate, %	18
Protein, %	31
Fat, %	53
Total vitamin A, RAE	2040 ± 941
β-Carotene, µg	6030 ± 3690
α-Carotene, µg	1080 ± 965
β-Cryptoxanthin, µg	221 ± 146
Lycopene, µg	7790 ± 3960
Lutein + zeaxanthin, µg	4060 ± 2860
Iron, mg	33.9 ± 24.0
Zinc, mg	25.4 ± 16.7

[a]Values are mean ± SD or percentage, $n = 25$.
RAE: retinol activity equivalents.[22]

Results

Subject data

Dietary intakes of the US women were quantified by the Harvard FFQ (Table 2).[22] All but two of the US women were consuming ≥1300 µg retinol activity equivalents (RAE)/d,

the recommended daily allowance for lactating women 19–50 years old, with a large proportion coming from β-carotene. Subject information to match serum and milk DR:R data, and information about the timing of the milk samples in the Indonesian trial were not retrievable. Therefore, serum DR:R values over time and breast milk retinol concentrations are reported separately for the Indonesian women.

Serum and breast milk retinol and carotenoids

Retinol concentration did not vary between serum and milk within either cohort or across time points in milk (US women) or serum (both cohorts) (Table 1, reported as the mean of all time); however, Indonesian women with a positive MRDR value $\geq$0.060 [VA-deficiency, 61% (11/18)] had lower serum retinol than those without (deficiency covariate $P < 0.01$). In the US women, 0% of serum samples and 32% (22/68) of milk samples were positive for VA deficiency by the WHO-prescribed cutoffs of 0.7 and 1.05 μmol retinol/L[4,23] for serum and milk, respectively, even though the DR:R indicated no deficiency in the US cohort. In the Indonesian women, 48% (26/54) of serum samples and 71% (17/24) of milk samples fell below these cutoffs. In Indonesian infants who were sampled at 24 or 48 h after their mothers were dosed with DRA, serum retinol and DR values were 0.45 ± 0.11 μmol/L and 5.8 ± 3.6 nmol/L, respectively, with serum retinol <0.7 μmol/L in 97% (28/29) of subjects. The mean DR:R in the infants, who only received DR from the breast milk and were not directly dosed, was measurable at 0.013 ± 0.012 (range: 0–0.058).

Serum and milk provitamin A and other selected carotenoids were determined in the US women (Table 3). The carotenoid profile of milk differed from that of serum and β-cryptoxanthin was enriched in milk compared with serum. US serum and milk retinol concentrations were not significantly different when comparing all time points among all individuals by paired *t*-test.

Serum and milk DR:R at different times post-dose

Serum and milk DR:R reached their maxima at 8 and 14 h post-dose, respectively, and declined thereafter in the US women (Figure 2(a) and (b)). In the Indonesian women, serum DR:R peaked between 6 and 12 h post-dose in VA-deficient and sufficient women, respectively (Figure 2(c)). The correlations in US women between milk and serum DR:R at 5, 6, and 7 h (times typically used for the serum MRDR test)[2,17] after administration of 8.8 μmol DRA were determined by linear regression (Figure 3(a) to (c)). The correlation was significant at 5 h ($P < 0.05$), but not at 6 or 7 h.

Serum and milk 3,4-didehydroretinol kinetics

Mean DR concentrations in serum (both groups) and milk (US women) were determined for each time point and the pooled group kinetics were analyzed by noncompartmental analysis. The Indonesian women were separated into VA-deficient and VA-sufficient according to DR:R as above for analysis; however, too few datapoints were available in the VA-sufficient women at 24 h so the noncompartmental analysis failed in this group. Subsequently, the US women's serum and milk DR kinetics, and the VA-deficient Indonesian women's serum DR kinetics were compared with previously determined DR kinetic values in VA-adequate and VA-depleted lactating sows given a larger DRA dose (35 μmol) more appropriate for their body weight (Table 4).[11,12] The VA-depleted sows were fed a VA-deficient diet for three gestation-lactation cycles, while the VA-adequate sows consumed a VA-adequate diet. VA-deficient sows had markedly different kinetics from the US women and the VA-adequate sows.[12]

Discussion

The MRDR test has been used in many countries to determine the prevalence of VA deficiency and adequacy in vulnerable groups, such as women and children.[2] The high prevalence of deficiency with the MRDR test and difference in response curves in these Indonesian women compared with the adequacy in the US women was important to report. Lactating women are an attractive target for VA status assessment and intervention due to the dual impact that VA deficiency may have on the woman and her infant.[24] For example, although two large maternal VA supplements given at birth and 24 h post-partum did not impact breast milk VA concentrations in Senegalese women at the time sampled, their infants' VA status at six months (measured by the MRDR test) was significantly

Table 3. Serum and breast milk carotenoids in US women 3–48 h after consuming 8.8 μmol 3,4-didehydroretinyl acetate.[a]

	Serum		Breast milk	
Carotenoid	μmol/L	% of total carotenoid	μmol/L	% of total carotenoid
β-Carotene	0.527 ± 0.337	21	0.064 ± 0.052	6
α-Carotene	0.157 ± 0.122	6	0.017 ± 0.022	2
β-Cryptoxanthin	0.304 ± 0.218	12	0.737 ± 0.730	72
Lycopene	0.909 ± 0.440	37	0.051 ± 0.035	5
Lutein	0.423 ± 0.198	17	0.140 ± 0.089	14
Zeaxanthin	0.160 ± 0.090	6	0.022 ± 0.029	2
Total carotenoid	2.480 ± 0.901	100	1.030 ± 0.816[b]	100[c]

[a]Values are mean ± SD, $n = 25$ (means of triplicate measures).
[b]Overall proportions of carotenoids in serum vs. milk were significantly different by χ^2 test ($P < 0.001$).
[c]Significantly different by paired *t* test ($P < 0.001$).

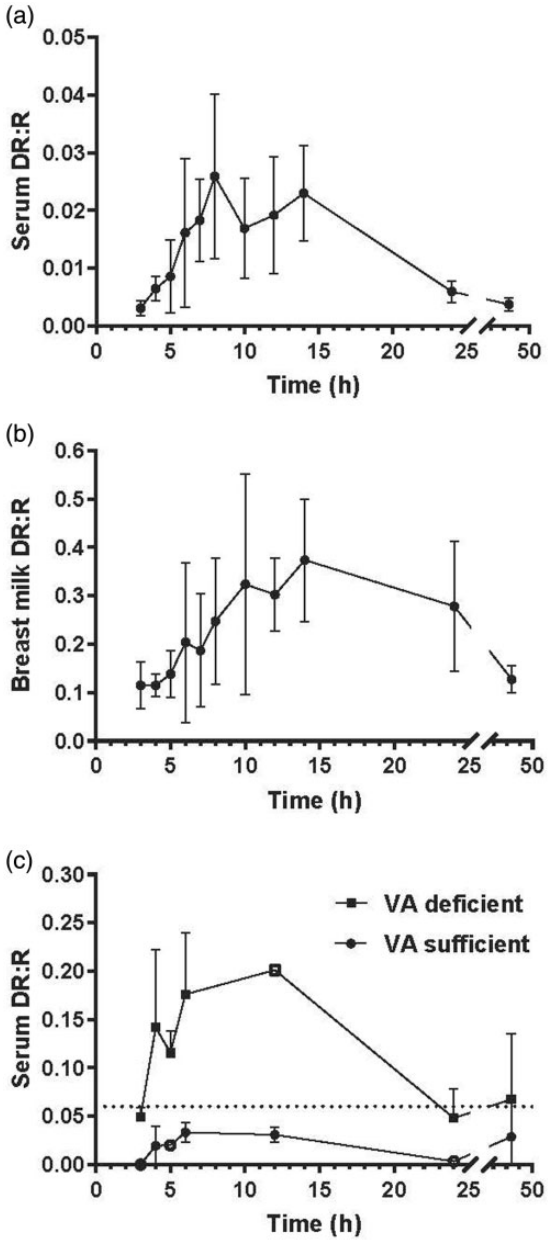

Figure 2. Time course of MRDR values (DR:R) in (a) serum and (b) breast milk in US lactating women and (c) serum in Indonesian lactating women (separated into deficient and sufficient as determined by MRDR $\geq$0.060 at 4–6 h) administered 8.8 μmol DRA. Values are mean ± SD, see Figure 1 for number of samples per time point (3 samples/woman). Open symbols indicate only a single sample for that time point. Note that the scale differs between serum and breast milk MRDR values. DR: 3,4-didehydroretinol; DRA: 3,4-didehydroretinyl acetate; MRDR: modified relative dose response; R: retinol.

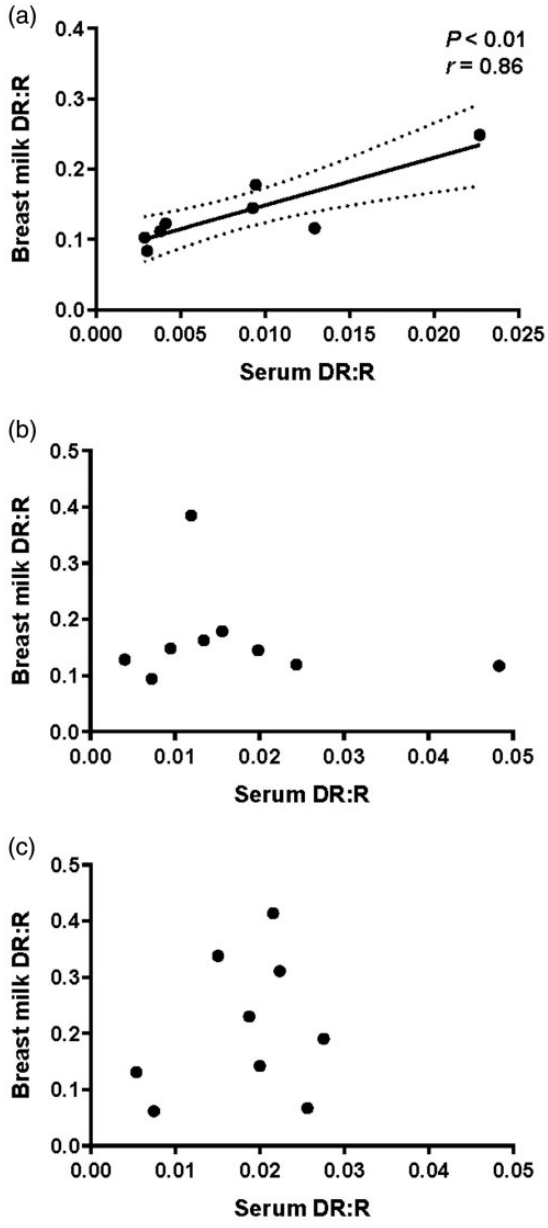

Figure 3. The relationship between the MRDR value (DR:R) in breast milk and serum in US lactating women at 5 h (a, $n = 8$), 6 h (b, $n = 9$), and 7 h (c, $n = 9$) following administration of 8.8 μmol DRA. The linear regression was significant at 5 h ($P < 0.01$, $r = 0.87$) with a slope (95% CI) of 6.8 (3.0–10.5) and y-intercept (95% CI) of 0.081 (0.041–0.012) but not significant at 6 or 7 h. DR: 3,4-didehydroretinol; DRA: 3,4-didehydroretinyl acetate; MRDR: modified relative dose response; R: retinol.

improved relative to infants from non-supplemented women.[25]

In this study, we obtained promising results on the use of a 5-h breast milk DR:R as a surrogate for serum DR:R in the MRDR test in US lactating women with adequate status, in line with results obtained from a lactating sow model.[11] A breast milk MRDR test represents an opportunity to expand VA status assessment by circumventing the aversion to blood draws that currently affects nutritional biomarker studies,[10] and thus warrants more comprehensive examination. The effect of blood draws on dropout rates is difficult to quantify in studies. A recent study in Tanzanian children using stable retinol isotope dilution (a VA status assessment technique that used two blood draws in this cohort)[2] recruited 52 participants at baseline but only 32 children returned for the d 14 blood draw, attributed in part to blood draw aversion (unpublished observations).

Breast milk sampling is generally considered to be accessible in most cultures, but should be explored in the local context.[23] Breast milk DR:R shows potential in responding to interventions, but further research in subjects undergoing milk collection at the same time as blood draw is required to determine variation in milk DR:R to generate

Table 4. Kinetic parameters of an 8.8 μmol oral dose of 3,4-didehydroretinol (DR) in US women's serum and breast milk and serum in vitamin A (VA) deficient Indonesian women.

	Maximum concentration (C_{max}) nmol/L	Time of C_{max} (T_{max})h	Half-life ($T_{1/2}$) h	Rate constant (λ_z) h^{-1}	$AUC_{0-48\ h}$[a] nmol/L × h	$AUC_{0-\infty}$[b] nmol/L × h
US women serum[c]	11.6	8	15.3	0.05	215	258
VA-deficient Indonesian women serum[d]	111	6	23.6	0.03	2290	3410
VA-adequate sow serum[e]	7.0 ± 2.5	7.5 ± 1.9	17.4 ± 4.5	0.04 ± 0.01	366 ± 193	570 ± 275
VA-depleted sow serum[e]	110 ± 130	6.4 ± 1.5	23 ± 11	0.04 ± 0.01	963 ± 662	1090 ± 693
US women breast milk[c]	139.3	12	22.4	0.03	3581	5078
VA-adequate sow milk[e]	292 ± 94.8	14.4 ± 9.8	71.8 ± 51.2	0.01 ± 0.006	9790 ± 4830	26800 ± 21200
VA-depleted sow milk[e]	870 ± 540	8.6 ± 7.0	8.6 ± 2.8	0.09 ± 0.03	14000 ± 6520	15300 ± 6990

[a]Area under the curve was determined by linear trapezoidal approximation from 0 to 48 with no absorption lag time.
[b]The additional area under the curve from 48 h to ∞ was determined by extending the log-scale terminal slope to a concentration of 0.
[c]Noncompartmental analysis of US women human data used mean DR concentration at each time point ($n = 25$ women, 4–10 women/time point, 3 samples/woman), yielding kinetic parameters for a single pooled model.
[d]Noncompartmental analysis of VA-deficient Indonesian women used mean DR concentration at each time point ($n = 11$ women, 1–5 women/time point, 3 samples/woman), yielding kinetic parameters for a single pooled model. Noncompartmental analysis of VA-sufficient was not possible because there were insufficient points to estimate a terminal slope.
[e]Noncompartmental analysis of sow data created an individual model for each sow, values represent mean ± SD, $n = 4$ (VA-adequate) or 7 (VA-depleted) for each parameter.[11,12]
Note: US women are compared with a 35 μmol dose in sows,[11,12] determined 3–48 h post-ingestion by noncompartmental analysis.
VA: vitamin A

a useful cutoff value for deficiency vs. sufficiency in humans. The serum and breast milk DR:R assays were used to evaluate a fortified cooking oil intervention in Indonesian lactating women compared with high-dose VA supplements.[26] Although there was a high percentage of VA deficiency before and after the intervention in these women by the serum MRDR test, both indicators were significantly different after the intervention due to a decrease in the DR:R values in the women who received both interventions and an increase in DR:R values (increased prevalence of VA deficiency) in the women who received the placebo. The serum DR:R had a much lower significance value indicating an intervention effect ($P = 0.003$)[26] than the milk DR:R ($P = 0.045$) (Permaesih D., personal communication), which may indicate that fewer women are needed when using serum samples for evaluation.

The milk MRDR values were 10-times those of serum in the US women, which may indicate that most DR in milk was delivered by chylomicra assembled in the intestines, which are not affected by the biological principle of the MRDR test (i.e. build-up of *apo*-RBP4 in the liver during VA-depletion). Because DR esters contained in lipoproteins are not measured in the serum MRDR assay with the fast throughput analytical method,[17] a 9 to 1 ratio of milk DR to serum DR could mean that up to 90% of milk DR was from chylomicra. Previous studies on milk VA secretion using acute (bolus) or chronic (dietary) VA intake have indicated that RBP4-derived VA can make up as much as 90% of milk VA in monkeys; however, with higher intakes, the proportion of VA from chylomicra increases significantly.[27]

Despite this, a spike in RBP4-derived DR can plausibly increase the DR:R beyond the ratio in serum for three reasons. First, milk DR:R was significantly higher in sows on a VA-deficient diet for three parities (VA-deficient) compared with two parities (VA-sufficient),[12] and was responsive to VA interventions in Indonesian women as noted above. Second, milk VA secretion is dynamic and responds to both chylomicra and RBP4 delivery. Two studies by O'Byrne *et al.*[28] examined milk VA in mice only able to transport VA in lipoproteins by knocking out RBP4 and in mice only able to source milk VA from RBP4 by knocking out lipoprotein lipase. If VA transport was static, the loss of one or the other pathways should decrease milk VA by the proportion that pathway contributes retinol to milk. However, in both studies, milk VA concentrations were not different from wild-type mice. This indicates that milk VA can be sourced entirely from RBP4, entirely from chylomicra, or a mixture of the two. Furthermore, VA-sufficient sows were administered 35 μmol α-retinol (a positional isomer of retinol that cannot bind RBP and therefore represents lipoprotein delivery alone)[29] or DR (as a tracer of both lipoprotein and RBP delivery). Both analog response curves in milk peaked at 0.3–0.4 μmol/L at similar times[11] despite the difference in delivery methods. Third, kinetic analysis in VA-deficient and sufficient sows,[12] and the VA-sufficient US women and VA-deficient Indonesian women demonstrated that milk and serum VA kinetics are markedly different between deficiency and sufficiency. It is possible that these kinetics are changing milk DR:R in a way that is not fully explained by hepatic RBP accumulation during VA deficiency. A future study could include feeding VA-deficient sows α-retinol to evaluate lipoprotein uptake during deficiency.

This study was limited because 23 of the 25 US women were consuming adequate dietary VA and all 25 women had adequate VA stores (serum MRDR values <0.060). Interestingly, several US women had milk retinol concentrations below the WHO cutoff for deficiency (<1.05 μmol/L)[23]; however, we have observed that following ingestion of a VA analog (i.e. α-retinol in sows (mentioned above)), retinol in milk was displaced by the incoming compound.[29] Many of the Indonesian women had deficient serum and milk retinol values in conjunction with the high rate of VA deficiency determined by their

DR:R. These data are reported to demonstrate serum DR:R in contrast to the US women. A complete study measuring the correlation of milk and serum DR:R in both VA-deficient and sufficient women is needed to confirm the previous results in VA-deficient sows to validate the breast milk measurements as a surrogate for serum.

The MRDR test has gained momentum as a surveillance biomarker due to the insensitivity of serum retinol concentrations to liver stores of VA.[2,5] Although these Indonesian women had a relatively low serum retinol concentration, the MRDR indicated an even higher prevalence of deficiency. In two groups of Ghanaian women, serum retinol concentrations were adequate (i.e. 1.4 ± 0.5 μmol/L and 1.5 ± 0.6 μmol/L)[30,31] but the MRDR values indicated a high level of deficiency in one cohort (DR:R 0.09 ± 0.05)[30] and not the other (0.048 ± 0.037).[31] In Indonesian lactating women, the MRDR test revealed a much higher prevalence of VA deficiency than serum retinol concentrations, and more sensitivity to response to VA supplementation.[15] The MRDR test better defines the underlying deficiency than serum retinol concentrations alone.[2]

An ancillary effect of using the MRDR test in lactating women is that the DR tracer is rapidly secreted into milk, transferred to the infant and measurable post-dose. This loss to milk supports the increased VA dietary needs for mothers during lactation. Researchers who want to perform the MRDR in infants should avoid using those from mothers who have been administered DR because there will be interfering DR in the suckling infants' serum from breast milk prior to the test. The measurability of DR:R in these infants who were not directly dosed supports using smaller doses for this age range in the MRDR test, such as the recent reduction to 3.5 μmol DRA in infants <2 years of age compared with the prior recommendation of 5.3 μmol DRA in all children <6 years old.[32]

In the US breast milk, we measured higher β-cryptoxanthin than that in serum and significant enrichment relative to other carotenoids. To our knowledge, this effect has not been observed in other studies.[33] The proportion of milk carotenoid tends to correlate with dietary intake on a national level without preference for β-cryptoxanthin,[34] so this was interesting given that the estimated intake of this carotenoid by the women was lower than the others profiled. β-Cryptoxanthin is unique among the major provitamin A carotenoids because of its structure as a xanthophyll; it is formed by the addition of a hydroxyl group to one β-ionone ring, which makes it hydrophilic.[35] This difference likely results in increased absorption in the intestine, which could also impact its uptake into mammary tissue from chylomicra and therefore preferential secretion into milk relative to other carotenoids, and higher apparent bioavailability of β-cryptoxanthin in humans have been reported.[36] The elevated concentration of β-cryptoxanthin also suggests adequate VA status in these US women because it was not cleaved to meet VA needs;[37] expression of the provitamin A carotenoid cleavage enzyme, β-carotene-15, 15′-oxygenase 1, is regulated by VA status.[38]

The DR:R in serum and breast milk was correlated in US women. The use of breast milk as a surrogate for the MRDR test in lactating women requires further validation in VA-deficient women prior to use in population evaluations. Standardization by sampling both blood and milk is necessary once inter-population variation is quantified. The serum DR:R was different between the US and Indonesian women and indicated a high level of deficiency in the Indonesian women. Further monitoring is needed especially because fortified cooking oil is now widely available in Indonesia.

Authors' contributions: JS analyzed data and wrote the paper; KB organized the data on the US women. TF assisted in the Indonesian study. SAT designed the studies, analyzed samples, and revised the manuscript. All authors read and approved the final manuscript.

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