Temporal Variation and Identification of Factors Associated with Endogenous Retinoic Acid Isomers in Serum from Brazilian Women

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Abstract

Objective: **Retinoids (natural and synthetic derivatives of vitamin A) have cancer chemotherapeutic and chemopreventive activities. Retinoic acid (RA) treatment has been associated with significant regression of preneoplastic lesions. However, serious toxicity associated with some therapies has made long-term chemoprevention in healthy populations unfeasible. Recently, serum RA has been shown to increase in response to oral retinol (vitamin A) supplementation. Here, we assess the variability of circulating RA levels and the lifestyle, demographic, and nutritional factors that explain such variability.**
Method: **Total RA concentration and the concentrations of RA isomers (all-*trans*-RA, 13-*cis*-RA, and 9-*cis*-RA) were measured by high-pressure liquid chromatography in serum samples obtained 4 months apart from 502 women participating in the Ludwig-McGill Cohort (São Paulo, Brazil).**
Results: **The relative abundance of the three RA isomers was similar for each visit (baseline and month 4), with 13-*cis*-RA having the highest concentrations followed by 9-*cis*-RA and all-*trans*-RA. The within-person variability of total RA and individual isomers was low. Using multivariate logistic regression models (upper tertile versus low/middle tertile of serum RA), we found that age, race, oral contraceptive use, total number of pregnancies, and season of initial blood draw were significantly associated with at least one endogenous RA isomer level. All endogenous RA isomers were positively associated with serum retinol, β-carotene, and β-cryptoxanthin levels.**
Conclusion: **These results have implications for the design of future epidemiologic studies focused on assessing RA-disease association and intervention studies aimed at modulating RA levels.** (Cancer Epidemiol Biomarkers Prev 2004;13(11):1693–703)

Introduction

Retinoic acid (RA) is required for many biological processes including vision, development, and reproduction (1-3). RA mediates these activities by binding to nuclear retinoid receptors, members of the steroid hormone receptor superfamily (4), and altering transcriptional activity (1, 5). To date, two families of receptors have been identified, RA receptor (RAR) and retinoid X receptor (5). RAR binds all-*trans*-RA and 9-*cis*-RA with equal affinity, whereas retinoid X receptor preferentially binds 9-*cis*-RA (6). Each receptor family has at least three subtypes (α, β, and γ), and within these subtypes, there are many different isoforms (7). Due to the different receptor isoforms, retinoid receptors can interact with a diverse number of receptors forming homodimers and heterodimers not only with retinoid receptors but also with other steroid hormone nuclear receptors such as vitamin D receptors and the estrogen receptor (8).

Through this potent activity as transcriptional regulators, retinoids (natural and synthetic derivatives of vitamin A) have been shown to be cancer chemotherapeutic and chemopreventive (see also refs. 5, 9, 10 for reviews). RA treatment (all-*trans*-RA) has been associated with significant regression of several preneoplastic lesions, including oral leukoplakia (11), cervical dysplasia (12), and actinic keratoses (5). Although retinoid chemoprevention is effective, there are toxicities associated with therapy such as varying degrees of teratogenicity and mucocutaneous cytotoxicity (5, 13). These toxicities make long-term chemoprevention among healthy populations with naturally occurring RA unfeasible. 13-*Cis*-RA (isotretinoin) and *N*-(4-hydroxyphenyl)retinamide (Fenretinide) have been shown to have lower toxicities, but it remains unclear if these drugs are able to induce retinoid receptor–mediated changes. An alternative retinoid chemoprevention approach is to develop a method for increasing endogenous concentrations of RA. Such an

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approach was shown to be feasible by Sedjo et al., (36) who reported the modulation of endogenous RA following chemopreventive doses of retinyl palmitate. The focus of this article is to examine if the variability of endogenous RA is associated with variability of dietary intake, implying that diet may modulate levels of RA safely.

Prior to developing research and programs to modulate endogenous RA levels, a reliable method for measuring endogenous RA levels is required and an understanding of the factors that influence endogenous RA levels is needed. For over a decade, various methodologies have been reported for the detection of RA in serum (14-24). Recent improvements in high-pressure liquid chromatography (HPLC) methodologies have facilitated the measurement of endogenous RA within the context of epidemiologic studies. The key improvements include an improved separation of three RA isomers and implementation of high-throughput methods. Using refined methodology for measuring circulating RA levels, it is now possible to assess the variability of RA and the factors associated with endogenous RA levels. This information is essential to the design of future epidemiologic studies focused on assessing RA-disease associations and intervention studies aimed at modulating RA levels.

Using blood samples collected 4 months apart in an ongoing prospective study, the Ludwig-McGill Cohort (São Paulo, Brazil), we investigated the variability of serum RA concentrations and its three major isomers (all-*trans*-RA, 13-*cis*-RA, and 9-*cis*-RA) over a 4-month period. We also examined whether certain lifestyle, demographic, and nutritional factors are associated with the variability in such measurements.

Materials and Methods

We used blood samples collected previously and stored as part of the Ludwig-McGill Cohort study of the natural history of human papillomavirus infection and cervical neoplasia (25). Briefly, from 1993 to 1997, the Ludwig-McGill Cohort enrolled a systematic sample of 2,528 women attending a comprehensive maternal and child health maintenance program catering to low-income families in the city of São Paulo. The clinic setting where participants were being accrued is part of a network of primary, secondary, and tertiary health care institutions maintained by the municipal health department. Cohort participants were examined every 4 months in the first year and twice yearly thereafter for a total of 5 years. At each study visit, participants were interviewed based on a structured questionnaire specific for the current visit (25). As described previously, the mean age at enrollment was 31 years (median, 33 years; ref. 25). The institutional review boards and ethical committees of all institutions with which the authors are affiliated approved these protocols. Each study participant signed an approved informed consent document.

Study Sample. Of the 2,528 women in the cohort, we identified a subcohort of women (n = 1,392), representing mostly those entering the study during the first 2 years (1993-1995) and who had provided long-term follow-up. We then selected the 846 women who provided data and serum for all four visits within the first year. Of these women, 810 had sufficient serum for RA analysis at visits 1 (month 0) and 2 (month 4). We then limited the study sample to those women that were human papillomavirus negative and had normal cervical cytology (n = 502).

Diet Questionnaire. Information on the frequency of consumption of selected food items and the consumption of vitamin and mineral supplements was obtained at visit 2 (month 4). This dietary questionnaire was specifically designed for application within the Ludwig-McGill Cohort to low-income women in São Paulo. Participants were asked to recall the usual frequency of consumption, during the past 5 years, of the following 15 food items: oranges, lemons, carrots, pumpkin, papaya, cauliflower, spinach, broccoli, lettuce, other vegetables, eggs, milk and yogurt, cheese, butter, and liver. These foods contributed substantially to variation in intake of carotenoids and tocopherols among Brazilian women living in São Paulo. The food consumption-frequency categories were as follows: never, <1 time monthly, 2 to 3 times monthly, 1 to 3 times weekly, 4-6 times weekly, and ≥1 time daily. Women in this study did not report a substantial consumption of vitamin and mineral supplements (26).

Nutrient Intake Values. Diet provides retinol (preformed vitamin A) in the form of retinol ester from animal products (liver, eggs, milk, cheese, and butter) or as pro–vitamin A carotenoids (e.g., β-carotene, α-carotene, and β-cryptoxanthin; refs. 1-3). Through a series of controlled metabolic reactions, retinol and pro–vitamin A carotenoids are converted to RA *in vivo*. Therefore, nutrient intake of vitamin A and carotenoid-rich foods was focused on in this report. The determination of nutrient values for this population has been reported in detail previously (26). In brief, nutrient values were calculated from the participants' reported dietary intake by use of the U.S. Food and Drug Administration's Continuing Survey of Food Intake of Individuals (1986) and Nationwide Food Consumption Survey (1987-1988), unless Brazil-specific nutrient values were available. When available, food carotenoid values (e.g., β-carotene, lutein/zeaxanthin, and β-cryptoxanthin) were derived from published values for foods consumed in São Paulo (27). Dietary intake calculations used age-specific portion sizes for women, as described elsewhere (28). From these databases, nutrient values were obtained for vitamin A, carotenoids (α-carotene, β-carotene, β-cryptoxanthin, and lutein/zeaxanthin), and vitamin C.

Because the questionnaire used in this study was short, we examined the association between consumption of certain fruits and vegetables and corresponding serum concentrations of carotenoids. Serum concentrations of certain carotenoids were correlated with consumption of citrus fruits and carrots (26) in a group of 100 women. In addition, we evaluated the crude and energy-adjusted (29) associations between intake of carotenoids and serum concentrations of carotenoids and found stronger associations when the energy-adjusted intake values for carotenoids were used (data not shown). The primary purpose of energy adjustment of nutrient values was to adjust for the tendency of individuals to consistently underreport or overreport intake values when a frequency checklist is used (29). Therefore, all dietary intake analyses used energy-adjusted nutrient values.

Table 1. Endogenous levels of RA, retinol, and β-carotene in Brazilian women (*n* = 502)

	Visit 1			Visit 2			P*
	Mean (SD)	Median	Range	Mean (SD)	Median	Range	
Total RA (ng/mL)	3.21 (1.23)	3.07	0.81-8.90	3.19 (1.33)	3.12	0.44-9.91	0.958
Cis-isomer (ng/mL)							
13-*Cis*-RA	1.35 (0.74)	1.21	0.15-5.60	1.30 (0.74)	1.25	0.15-7.00	0.604
9-*Cis*-RA	1.02 (0.58)	1.06	0.15-3.90	1.05 (0.59)	1.08	0.15-3.83	0.445
All-*trans*-isomer	0.83 (0.39)	0.76	0.15-3.50	0.83 (0.45)	0.76	0.15-5.14	0.810
Retinol (μg/mL)	0.465 (0.169)	0.437	0.007-2.083	0.446 (0.172)	0.416	0.075-1.998	0.012
Total β-carotene (μg/mL)	0.044 (0.079)	0.020	0.004-0.650	0.032 (0.039)	0.021	0.004-0.609	0.762

*Ps testing the null hypothesis that the difference between visits 1 and 2 is zero using the Wilcoxon rank sum test.

Sample Processing and Storage. All nonfasting blood samples (~10 mL) were collected by venipuncture into green top vacutainers by a trained nurse at the time of the clinic visit. The samples were centrifuged within 6 to 8 hours of collection. Aliquots (1 mL) of serum were stored in 1.8 mL Nunc cryovials at −20°C in a non-frost-free freezer until shipped for analyses. Serum from the first two clinical visits (months 0 and 4) was used to measure RA. To address the known variability of serum carotenoids (30), we determined serum retinol and carotenoids in four serum samples obtained within the first year (months 0, 4, 8, and 12). Total cholesterol was determined at the third visit (month 8) by coupled enzymatic, colorimetric assay (Sigma kit 401-25P, Sigma Chemical Co., St. Louis, MO).

Serum RA HPLC Analytic Procedures. RA analyses were conducted on serum samples from the first two visits (months 0 and 4). The 9-*cis*-RA and all-*trans*-RA were purchased from Sigma Chemical and 13-*cis*-RA was obtained from ICN Biomedicals (Aurora, OH). The following reagents were used for sample preparation and analysis: butylated hydroxytoluene, hexane, ethanol, methanol, acetonitrile, HCl, NaOH, and acetic acid. All solvents were HPLC grade or equivalent and were used without further treatment.

Sample extraction and preparation was done using a modification of the method reported by Miyagi et al. (14). After thawing 500 μL aliquots of serum, samples were deproteinated with 500 μL of acetonitrile/methanol (19:1) containing 0.01% butylated hydroxytoluene and made alkaline with 100 μL of 2 N NaOH. Samples were extracted by vortex mixing for 45 seconds with 1.5 mL of hexane containing 0.025% butylated hydroxytoluene as an antioxidant. The organic phase was discarded. Samples were acidified with 200 μL of 2 N HCl and extracted three times with 1.5 mL of hexane with butylated hydroxytoluene. The combined supernatant was evaporated under nitrogen. The residue was dissolved by vortex mixing with 120 μL of mobile phase consisting of 75% acetonitrile, 5% methanol, and 20% of 1% acetic acid. The injection volume was 90 μL.

The HPLC separation was done using a modified method reported by Dimitrova et al. (15). HPLC analysis was done using a ThermoSeparation Products liquid chromatograph with the following components: P4000 solvent delivery system, vacuum degasser, AS3000 autosampler, Spectra FOCUS scanning UV-visible detector, and PC1000 computer-controlled data system (Fremont, CA). On the autosampler, samples were refrigerated at 10°C and the column was maintained at 30°C. RA isomers were monitored at 350 nm. The analytic column was a Spherisorb ODS2 (3 μm, 4.0 × 250 mm) with Javelin guard column containing Keystone ODS2 (3 μm, Keystone Scientific, Inc., Bellefonte, PA). The mobile phase had a flow rate of 1 mL/min.

Linear calibration curves were prepared consisting of three concentrations of RA isomers that spanned the physiologic levels in serum. Quantification was done by external standard calibration using peak area ratios. In-house quality control samples were analyzed at the beginning and end of each sample queue. The relative SD of analytes in the quality control samples ranged from 10% to 15%. This system had a limit of detection of 0.1 ng/mL and limit of quantification of 0.3 ng/mL (5 pmol/L). Samples below the limit of quantification were assigned a value halfway between zero and the lower limits of detection: these accounted for 10 (1%) all-*trans*-RA, 20 (2%) 13-*cis*-RA, and 85 (8.5%) 9-*cis*-RA samples of the 1,004 samples analyzed.

Serum Retinol and Carotenoids HPLC Analytic Procedures. Archival serum from all four visits within the first year (months 0, 4, 8, and 12) on study were

Table 2. Spearman correlation coefficients, between-person and within-person variances, and between-person to within-person ratios of serum retinoids

	Spearman correlation*	n†	Between-person variance‡	Within-person variance	Between-person/within-person ratio
Total RA (ng/mL)	0.67	502	0.122	0.068	1.796
Cis-isomer (ng/mL)					
13-*Cis*-RA	0.57	480	0.139	0.132	1.053
9-*Cis*-RA	0.79	424	0.165	0.053	3.092
All-*trans*-isomer	0.56	485	0.100	0.086	1.161

*All $P < 0.001$.
†Random effects models were restricted to data above limit of quantitation; n varies by nutrient. Spearman correlation coefficient includes 502 women.
‡Log-transformed nutrient values used in random effects analysis.

Table 3. Serum total RA and RA isomer concentrations in women stratified by lifestyle and demographic characteristics (mean RA concentration at visits 1 and 2 per individual)

	Median concentration (ng/mL)				
	n	Total RA	13-*Cis*-RA	9-*Cis*-RA	All-*trans*-RA
Age (y)					
<20	21	2.75	1.22	1.04	0.61
20-29	198	2.84	1.11	0.91	0.74
30-39	176	3.46	1.45	1.22	0.78
≥40	106	3.39	1.32	1.21	0.79
P_{trend}*		<0.001	<0.001	<0.001	0.04
Race					
White	328	3.19	1.30	1.12	0.79†
Non-White	173	3.07	1.28	1.07	0.71
Cigarette smoking					
Never	277	3.09‡	1.22†	1.09†	0.74
Current	147	3.20	1.33	1.13	0.79
Former	77	3.24	1.33	1.11	0.76
Alcohol (glass/wk)					
Never	174	3.20	1.37	1.10	0.74
≤1	262	3.10	1.25	1.07	0.79
>1	66	3.25	1.33	1.22	0.74
Years consuming alcohol					
Never	174	3.20	1.37	1.10	0.74
≤5	113	3.18	1.35	1.10	0.70
6-10	84	2.88	1.06	1.00	0.85
11-15	38	3.25	1.25	1.14	0.90
>15	73	3.36	1.41	1.20	0.78
P_{trend}		0.78	0.81	0.80	0.06
OC use (y)					
Never	78	3.16	1.41	1.07	0.73
<6	276	3.06	1.20	1.06	0.74
≥6	147	3.40	1.39	1.20	0.80
P_{trend}		0.10	0.53	0.18	0.07
Total no. pregnancies					
0-1	87	2.87	1.11	1.01	0.72
2-3	214	3.02	1.20	1.06	0.73
≥4	199	3.47	1.41	1.18	0.80
P_{trend}		<0.001	<0.001	<0.001	0.05
Education					
Illiterate	35	3.19	1.33	1.23	0.74
<Elementary	77	3.24	1.30	1.08	0.76
Elementary	283	3.10	1.29	1.10	0.75
>High school	43	3.11	1.35	1.07	0.70
≥High school	63	3.26	1.38	1.13	0.84
P_{trend}		0.76	0.94	0.28	0.61
Income (US$)					
<250	126	2.81	1.05	1.00	0.82
250-450	123	3.13	1.20	1.07	0.75
451-725	118	3.19	1.33	1.15	0.79
>725	118	3.30	1.41	1.17	0.69
P_{trend}		<0.001	<0.001	<0.001	0.02
Total no. household members					
1-3	108	3.15	1.25	1.10	0.87
4	148	3.10	1.30	1.07	0.73
5	119	3.28	1.30	1.13	0.73
≥6	126	3.11	1.32	1.10	0.73
P_{trend}		0.99	0.34	0.96	0.04

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Table 3. Serum total RA and RA isomer concentrations in women stratified by lifestyle and demographic characteristics (mean RA concentration at visits 1 and 2 per individual) (Cont'd)

Season§					
Fall	198	3.20‖	1.38‖	1.10‖	0.82‖
Winter	95	3.19	1.21	1.20	0.72
Spring	99	3.10	1.31	1.05	0.62
Summer	110	2.55	0.85	0.81	0.83
Total serum cholesterol (mg/dL)					
44-134	126	2.83	1.19	0.99	0.73
135-160	118	3.28	1.38	1.11	0.73
161-188	127	3.13	1.25	1.13	0.73
189-386	129	3.37	1.34	1.20	0.84
P_{trend}		<0.001	0.01	<0.001	0.08
Tertiles of serum retinol (μg/mL)¶					
Low (0.165-0.392)	165	2.88	1.19	1.00	0.71
Medium (0.393-0.478)	177	3.19	1.33	1.12	0.73
High (0.479-1.550)	160	3.46	1.40	1.22	0.88
P_{trend}		<0.001	<0.001	<0.001	<0.001
Tertiles of serum β-carotene (μg/mL)¶					
Low (0.004-0.017)	165	2.98	1.27	1.05	0.67
Medium (0.018-0.031)	159	3.22	1.32	1.15	0.82
High (0.032-0.287)	178	3.33	1.30	1.17	0.83
P_{trend}		0.26	0.69	0.21	<0.001

*Nonparametric test for linear trend across ordered groups.
†$P < 0.05$, differences in RA concentration using Wilcoxon rank sum test (2 categories) or the Kruskal-Wallis test (≥3 categories).
‡$P < 0.01$, differences in RA concentration using Wilcoxon rank sum test (2 categories) or the Kruskal-Wallis test (≥3 categories).
§For season of blood sampling, only the visit 1 concentrations were shown.
‖$P < 0.001$, differences in RA concentration using Wilcoxon rank sum test (2 categories) or the Kruskal-Wallis test (≥3 categories).
¶Tertiles distribution of mean of four measurements per women within 12 months.

analyzed to detect retinol and carotenoids in serum using a modification of the procedures described by Nomura et al. (31). The percentage coefficient of variation was ≤8% for retinol and all carotenoids measured. This HPLC system's limit of quantification was 0.004 μg/mL for the carotenoids. Of the total 2,008 samples analyzed over four clinical visits, the following numbers of samples (%) were below the limit of quantification and assigned a value halfway between zero and the lower limits of detection: retinol 2 (<0.1%), α-cryptoxanthin 654 (33%), β-cryptoxanthin 187 (9%), α-carotene 514 (26%), *trans*-β-carotene 296 (15%), *cis*-β-carotene 621 (30%), lutein 10 (0.5%), and lycopene 425 (21%). Due to many samples being below the detectable limit for α-cryptoxanthin and α-carotene, these results will not be used in the analyses. *Trans*-β-carotene and *cis*-β-carotene were combined for a measure of total serum β-carotene.

Statistical Analysis. RA concentrations were right skewed; therefore, we used nonparametric statistical methods for most analyses. Differences between month 0 (visit 1) and month 4 (visit 2) were assessed using a Wilcoxon rank sum test, and correlations between paired nutrient values were examined by calculating Spearman correlation coefficients. To assess the between-woman and within-woman variability of RA, we estimated a linear mixed effects model using log-transformed nutrient values. Only samples that were above the detectable limit of the assay were included in this analysis. The mixed models analyses were done using PROC MIXED in SAS version 9.0 (Cary, NC; ref. 32).

As the differences in RA concentrations were low between visits (Table 1), we calculated the mean of two RA measures per individual. We determined if there was a difference in the rank distribution of total RA and each isomer by lifestyle and demographic characteristics using the Wilcoxon rank sum test (2 categories) or Kruskal-Wallis test (≥3 categories) without regard to the ordering. For factors that were ordered, a nonparametric test for trend, which is an extension of the Wilcoxon rank sum test (33), was used to test whether there was a significant trend across ordered groups. Spearman correlations were calculated between total RA and each isomer, serum retinol, and serum β-carotene for each visit, adjusting for laboratory batch analysis.

Multiple logistic regression was done to estimate the magnitude of the associations [odds ratio (OR) and 95% confidence interval (95% CI)] with RA levels and various sociodemographic and lifestyle factors. The study aim was to determine factors independently associated with elevated levels of RA, total and isomer specific. Therefore, we categorized women as having high values if their endogenous RA level fell within the upper tertile of the overall distribution and women as having low to medium levels if their level was below the upper tertile. Modeling began by using backward stepwise logistic regression models to identify the lifestyle and demographic factors that were independently associated with total RA or individual isomers with a probability of removal set at 0.1. Factors that were considered in the first set of models were race (White versus non-White), oral contraceptive (OC) use, total number of pregnancies, smoking status, alcohol consumption, season of enrollment, education, and income. Age, in intervals of 5 years, was included in all final models regardless of significance level. A stabilization of the Brazilian economy

Table 4. Associations of total RA and RA isomers with lifestyle and demographic factors among Brazilian women

	Total RA (n = 451)*				13-*Cis*-RA (n = 451)*			
	Low-medium RA (n = 281)	High RA (n = 170)	Crude OR†	Adjusted OR‡ (95% CI)	Low-medium RA (n = 282)	High RA (n = 169)	Crude OR†	Adjusted OR‡ (95% CI)
Age, 5 y (mean ± SD)	29 ± 8.3	35 ± 8.5	1.42	1.34 (1.11-1.62)	30 ± 8.6	33 ± 8.5	1.07	1.05 (0.89-1.23)
Race								
White	180	111	1.00		177	114	1.00	
Non-White	101	59	0.98		105	55	0.81	
Season (visit 1)								
Fall	105	77	1.00		97	80	1.00	1.00 (Reference)
Winter	61	34	1.03		61	34	0.67	0.68 (0.32-1.44)
Spring	60	32	0.58		65	27	0.34	0.36 (0.15-0.85)
Summer	55	18	0.65		60	23	0.42	0.45 (0.22-0.92)
OC use (y)								
Never	41	31	1.00	1.00 (Reference)	43	29	1.00	
<6	172	74	0.50	0.47 (0.24-0.91)	162	84	0.64	
>6	68	65	1.17	0.89 (0.43-1.83)	77	56	0.93	
Total no. pregnancies								
0-1	61	16	1.00	1.00 (Reference)	55	22	1.00	
2-3	125	66	2.26	1.90 (0.91-3.97)	127	64	1.31	
>4	95	88	3.48	2.42 (1.13-5.18)	100	83	1.75	

*Cut point between low-medium and high RA levels: total RA, 3.65 ng/mL; 13-*cis*-RA, 1.53 ng/mL ; 9-*cis*-RA, 1.30 ng/mL; and all-*trans*-RA, 0.92 ng/mL.
† Crude models are adjusted for laboratory analysis batch.
‡ Adjusted simultaneously for all variables for each specific RA isomer, laboratory analysis batch, and economy.

occurred in July 1994, 9 months following initial enrollment in the study, which most likely effected women's socioeconomic status and spending patterns. We found this to be associated with differences in serum carotenoids and controlled for this confounding variable by inputting an indicator variable (enrollment before July 1994 or after) into the final multivariate models. Finally, a difference in chromatographic results across HPLC run batches was observed. This was controlled statistically by including a variable for HPLC batch in all analyses. However, because the RA category was the same for all samples in two batches, these data were dropped during statistical modeling (n = 49 and 107, respectively). As a result, a smaller number of women were included in these logistic regression models (n = 451 for 13-*cis*-RA, 393 for 9-*cis*-RA, and 501 for all-*trans*-RA). To assess the influence of these two batches on our modeling, we repeated the logistic regression analyses removing all samples from the specified batches and found no differences in our conclusions (data not shown).

Using the above as a base model, we examined whether serum retinol and carotenoids (pro–vitamin A: β-carotene, α-carotene, β-cryptoxanthin, and lutein and non-pro–vitamin A: lycopene) were independently associated with RA levels using multivariate logistic regression. In addition, we examined if RA levels were associated with increased vitamin A and pro–vitamin A carotenoid intake and consumption of select foods. To test for linear trends, we treated all nutrient variables as continuous in the multivariate logistic regression models. Statistical significance was set at the 0.05 level and all analyses were two sided. Univariate and logistic regression analyses were done in Stata version 7.0 (College Station, TX), and mixed models analyses and adjusted Spearman correlations were done in SAS version 9.0 (34).

Results

The mean, median, and range of the concentrations of endogenous RA, retinol, and β-carotene at each visit are presented in Table 1. Overall, total RA concentrations ranged from 0.81 to 8.90 ng/mL (2.70-29.6 nmol/L) at visit 1 and from 0.44 to 9.91 ng/mL (1.46-32.98 nmol/L) at visit 2 and did not differ by visit. Median (range) baseline endogenous concentrations among the 502 women were 1.30 (0.15-4.90), 1.10 (0.15-3.23), and 0.76 (0.15-3.17) ng/mL for 13-*cis*-RA, 9-*cis*-RA, and all-*trans*-RA, respectively. The relative abundance of the three RA isomers was similar for each visit, with 13-*cis*-RA having the highest concentrations followed by 9-*cis*-RA and all-*trans*-RA. Median serum retinol levels were significantly lower at visit 2 compared with visit 1. However, when assessed over four available visits, no significant difference in retinol across visits was observed (P = 0.08; data not shown).

Spearman correlation coefficients for total RA, RA isomers, retinol, and β-carotene between visits 1 and 2 are shown in Table 2. Total endogenous RA level over a 4-month period was highly correlated (Spearman ρ = 0.67), as were the three isomers, with 9-*cis*-RA having the highest correlation (Spearman ρ = 0.79). The within-person variability of total RA and individual isomers was low, with the between-person variance to within-person variance ratios ranging from 1.05 for 3.09 (Table 2).

We also examined RA isomer concentrations by lifestyle and demographic factors using the mean concentration of visits 1 and 2 for each woman (Table 3). Total RA and its isomers increased with age (P_{trend} < 0.001, all-*trans*-RA P_{trend} < 0.05), increasing number of pregnancies (P_{trend} < 0.001, all-*trans*-RA P_{trend} < 0.05), and increasing level of serum retinol (P_{trend} < 0.001). Median 13-*cis*-RA and 9-*cis*-RA levels increased with increasing income (P_{trend} < 0.001), whereas all-*trans*-RA

Table 4. Associations of total RA and RA isomers with lifestyle and demographic factors among Brazilian women (Cont'd)

9-*Cis*-RA (*n* = 393)*				All-*trans*-RA (*n* = 501)*			
Low-medium RA (*n* = 233)	High RA (*n* = 160)	Crude OR†	Adjusted OR‡ (95% CI)	Low-medium RA (*n* = 334)	High RA (*n* = 168)	Crude OR†	Adjusted OR‡ (95% CI)
29 ± 8.5	34 ± 8.3	1.57	1.62 (1.34-1.95)	31 ± 8.6	32 ± 8.6	1.00	0.95 (0.81-1.12)
140	115	1.00	1.00 (Reference)	211	117	1.00	
93	45	0.58	0.63 (0.39-1.02)	123	50	0.65	
89	65	1.00		112	86	1.00	1.00 (Reference)
43	39	1.18		74	21	0.43	0.44 (0.21-0.91)
60	32	0.64		82	17	0.35	0.29 (0.12-0.72)
42	24	0.74		66	44	0.66	0.86 (0.47-1.58)
38	27	1.00		55	23	1.00	
135	76	0.72		187	89	1.17	
60	57	1.31		92	55	1.43	
47	22	1.00		64	23	1.00	1.00 (Reference)
100	61	1.28		147	67	1.16	1.19 (0.61-2.33)
86	77	1.84		123	76	1.67	1.89 (0.93-3.85)

decreased with increasing income ($P_{\text{trend}} = 0.02$). For all factors examined, results were similar when RA concentrations were evaluated for visits 1 and 2 separately rather than as the mean (data not shown). Median total RA and two isomers (13-*cis*-RA and 9-*cis*-RA) differed by cigarette smoking status (never, current, or former). Median all-*trans*-RA was higher among White women compared with non-White women.

Table 4 presents results of multivariate modeling of lifestyle and demographic factors independently associated with RA levels. Age, measured in 5-year intervals, was positively associated with total RA and 9-*cis*-RA levels (adjusted OR, 1.34; 95% CI, 1.11-1.62 and adjusted OR, 1.62; 95% CI, 1.34-1.95, respectively). Season of blood draw was significantly associated with 13-*cis*-RA and all-*trans*-RA (spring versus fall, adjusted OR, 0.36; 95% CI, 0.15-0.85 and adjusted OR, 0.29; 95% CI, 0.12-0.72, respectively).

Table 5 presents independent associations between nutritional factors and serum RA. There were strong, positive associations among all retinoids and serum retinol, β-carotene, and β-cryptoxanthin. Consistent with the proposed RA metabolism, carotenoids lacking the appropriate ring structure, such as lycopene, were not associated with serum RA isomer levels. In addition, RA isomer levels were not associated with serum lutein. These results are key in supporting the specificity of the associations found with serum RA isomers and select pro–vitamin A carotenoids. The associations between intake of specific nutrients and circulating concentrations of RA isomers are also reported in Table 5. Increased intake of vitamin A was significantly positively associated with 13-*cis*-RA (adjusted OR, 2.65; 95% CI, 1.48-4.73) and 9-*cis*-RA (adjusted OR, 1.85; 95% CI, 1.04-3.27) and marginally associated with total RA (adjusted OR, 1.74; 95% CI, 0.98-3.08). Increased intake of β-carotene was positively associated with 13-*cis*-RA (adjusted OR, 1.78; 95% CI, 1.01-3.12). Increased consumption of liver, pumpkin, and oranges was significantly positively associated with RA levels (total RA, 13-*cis*-RA, or 9-*cis*-RA) and may be the contributing sources for the positive associations shown with vitamin A and β-carotene intake and serum RA levels (data not shown).

Discussion

We characterized total serum RA and three individual RA isomers (13-*cis*-RA, 9-*cis*-RA, and all-*trans*-RA) in a large sample of women and determined factors associated with these levels. The relative abundance of the three RA isomers was similar for each visit, with 13-*cis*-RA having the highest concentrations followed by 9-*cis*-RA and all-*trans*-RA. Overall, we observed a wide range of values for this population (total RA 0.71-7.79 ng/mL), which may reflect the heterogeneity of this sample of low-income Brazilian women. Whereas the between-person variability was high, we observed low within-person variability of two measures of serum RA obtained ~4 months apart.

Sociodemographic factors were related to RA level. Age, race, OC use, total number of pregnancies, and season of initial blood draw were significantly associated with endogenous RA concentrations; however, factors significantly contributing to RA levels differed for each isomer. All endogenous RA isomers assessed in this study were positively associated with serum retinol, β-carotene, and β-cryptoxanthin levels. Associations were confirmed by observing significant associations with preformed vitamin A intake and increased consumption of pro–vitamin A–containing foods. Consistent with the proposed RA metabolism, carotenoids lacking the appropriate ring structure, such as lycopene, were not associated with serum RA isomer levels. These associations differed slightly for each RA isomer, suggesting the possibility of independent mechanisms for modulating specific isomer level. Additional laboratory research and population studies are required to define the mechanisms leading to RA isomerization and factors that modify this process. Now, it seems that the measurement

Table 5. Association of total RA and RA isomers and nutritional factors

	Total RA (ng/mL; n = 451)				13-*Cis*-RA (ng/mL; n = 451)			
	Low-medium RA	High RA	Crude OR*	Adjusted† OR (95% CI)	Low-medium RA	High RA	Crude OR*	Adjusted† OR (95% CI)
A. Circulating nutrients								
Serum retinol (μg/mL)								
Low (0.165-0.392)	103	37	1.00	1.00	95	45	1.00	1.00
Medium (0.393-0.478)	98	66	2.30	2.18 (1.20-3.95)	104	60	1.27	1.34 (0.76-2.36)
High (0.479-1.550)	78	68	3.38	3.32 (1.74-6.32)	83	63	2.04	2.30 (1.26-4.19)
P_{trend}				0.001				0.006
Serum β-carotene (μg/mL)								
Low (0.165-0.392)	93	51	1.00	1.00	93	51	1.00	1.00
Medium (0.393-0.478)	97	46	1.07	0.99 (0.53-1.84)	95	48	0.89	0.84 (0.45-1.56)
High (0.479-1.550)	62	68	4.23	4.58 (2.12-9.89)	69	61	3.04	3.17 (1.48-6.77)
P_{trend}				0.001				0.008
Serum β-cryptoxanthin (μg/mL)								
Low (0.002-0.012)	116	44	1.00	1.00	107	53	1.00	1.00
Medium (0.013-0.029)	90	61	2.14	1.97 (1.09-3.56)	95	56	1.21	1.20 (0.68-2.14)
High (0.03-1.47)	73	66	4.19	3.76 (1.89-7.48)	80	59	2.04	2.24 (1.14-4.42)
P_{trend}				0.001				0.022
Serum lutein (μg/mL)								
Low (0.009-0.027)	109	46	1.00	1.00	102	53	1.00	1.00
Medium (0.028-0.040)	90	63	1.45	1.38 (0.78-2.45)	89	63	1.38	1.21 (0.69-2.11)
High (0.041-0.219)	80	63	2.22	2.12 (1.17-3.83)	91	52	1.41	1.35 (0.76-2.41)
P_{trend}				0.013				0.360
Serum lycopene (μg/mL)								
Low (0.002-0.0093)	109	52	1.00	1.00	108	53	1.00	1.00
Medium (0.0094-0.018)	92	60	1.29	1.15 (0.65-2.04)	93	59	1.05	1.06 (0.60-1.87)
High (0.0181-0.1203)	78	59	1.72	1.56 (0.77-3.16)	81	56	1.53	1.55 (0.77-3.11)
P_{trend}				0.230				0.253
B. Energy-adjusted nutrient intake value								
Vitamin A (retinol equivalents)								
Low	109	41	1.00	1.00	110	40	1.00	1.00
Medium	86	65	1.99	1.90 (1.07-3.38)	89	62	2.45	2.33 (1.31-4.17)
High	86	65	1.90	1.74 (0.98-3.08)	84	67	2.75	2.65 (1.48-4.73)
P_{trend}				0.063				0.001
α-Carotene (μg/mg)								
Low	91	57	1.00	1.00	96	52	1.00	1.00
Medium	95	58	0.95	0.89 (0.51-1.57)	100	53	1.02	0.97 (0.56-1.69)
High	95	56	0.80	0.70 (0.40-1.24)	87	64	1.25	1.25 (0.72-2.17)
P_{trend}				0.227				0.419
β-Carotene (μg/mg)								
Low	95	54	1.00	1.00	101	48	1.00	1.00
Medium	91	59	1.26	1.21 (0.68-2.13)	93	57	1.56	1.57 (0.88-2.78)
High	95	58	1.11	1.07 (0.61-1.90)	89	64	1.90	1.78 (1.01-3.12)
P_{trend}				0.826				0.049
β-Cryptoxanthin (μg/mg)								
Low	98	51	1.00	1.00	94	55	1.00	1.00
Medium	94	58	1.11	1.02 (0.57-1.81)	100	51	0.97	0.97 (0.55-1.70)
High	89	63	1.49	1.39 (0.79-2.48)	89	63	1.41	1.39 (0.80-2.41)
P_{trend}				0.249				0.232
Lutein (μg/mg)								
Low	97	52	1.00	1.00	94	55	1.00	1.00
Medium	97	55	1.06	1.00 (0.56-1.78)	104	48	0.92	0.93 (0.53-1.63)
High	87	64	1.51	1.44 (0.81-2.54)	85	66	1.56	1.57 (0.90-2.72)
P_{trend}				0.206				0.101

*Crude model adjusted for analysis batch.

†Logistic regression model adjusted simultaneously for associated demographic and lifestyle variables (Table 4), laboratory analysis batch, economy, and cholesterol (circulating nutrients only).

of multiple isomers of RA within epidemiologic studies provides added information compared with only assessing total endogenous RA level.

The observed concentrations of 13-*cis*-RA in these women are similar to reports published previously (16-19). Soderlund et al. (16) reported an average 13-*cis*-RA concentration of 1.35 ng/mL (range 1.26-1.5) in a small sample of northern European women (mean age, 36 years). Others reported higher values (20-22) of 13-*cis*-RA, which may reflect differences in study participants (e.g., diet, age, sex, or presence of benign disease) or laboratory methods used. Sedjo et al. (36) assessed baseline levels of RA concentrations in a chemoprevention trial and found a relative abundance of each isomer similar to what we observed in this study (13-*cis*-RA > 9-*cis*-RA > all-*trans*-RA).

Table 5. Association of total RA and RA isomers and nutritional factors (Cont'd)

9-*Cis*-RA (ng/mL; n = 393)				All-*trans*-RA (ng/mL; n = 501)			
Low-medium RA	High RA	Crude OR*	Adjusted† OR (95% CI)	Low-medium RA	High RA	Crude OR*	Adjusted† OR (95% CI)
83	31	1.00	1.00	119	45	1.00	1.00
80	63	2.73	2.55 (1.43-4.54)	122	55	1.54	1.65 (0.93-2.95)
69	66	4.21	3.84 (2.08-7.09)	92	67	3.34	4.04 (2.16-7.54)
			0.001				0.001
101	40	1.00	1.00	110	40	1.00	1.00
74	46	1.50	1.31 (0.72-2.38)	97	57	1.83	1.82 (0.98-3.39)
36	67	4.98	4.86 (2.22-10.63)	100	60	2.08	3.04 (1.42-6.55)
			0.001				0.004
111	40	1.00	1.00	124	44	1.00	1.00
81	51	1.56	1.22 (0.69-2.16)	107	59	1.63	1.82 (1.03-3.23)
40	69	4.32	3.21 (1.65-6.26)	102	64	1.82	2.39 (1.23-4.64)
			0.001				0.009
92	45	1.00	1.00	119	49	1.00	1.00
78	56	1.27	1.07 (0.61-1.89)	109	58	1.10	1.18 (0.68-2.07)
62	59	1.85	1.55 (0.86-2.79)	105	60	1.23	1.57 (0.89-2.76)
			0.147				0.120
104	45	1.00	1.00	120	52	1.00	1.00
82	54	1.44	1.37 (0.78-2.42)	102	60	1.35	1.31 (0.75-2.27)
46	61	2.00	1.70 (0.83-3.49)	111	55	0.93	1.15 (0.59-2.25)
			0.129				0.580
97	40	1.00	1.00	122	46	1.00	1.00
71	58	1.78	1.70 (0.96-3.02)	105	62	1.18	1.17 (0.68-2.02)
66	62	2.00	1.85 (1.04-3.27)	107	60	1.07	1.04 (0.60-1.81)
			0.038				0.917
80	52	1.00	1.00	111	57	1.00	1.00
75	56	1.13	0.94 (0.54-1.66)	106	61	1.23	1.19 (0.70-2.03)
79	52	0.89	0.78 (0.44-1.38)	117	50	1.10	0.72 (0.42-1.25)
			0.391				0.245
77	58	1.00	1.00	119	49	1.00	1.00
77	51	0.86	0.73 (0.41-1.29)	107	60	1.12	1.23 (0.71-2.13)
80	51	0.84	0.71 (0.41-1.26)	108	59	0.72	1.08 (0.63-1.85)
			0.252				0.820
76	53	1.00	1.00	118	50	1.00	1.00
76	58	1.13	0.96 (0.55-1.69)	112	55	1.10	1.08 (0.63-1.87)
82	50	0.91	0.73 (0.41-1.31)	104	63	1.56	1.60 (0.93-2.77)
			0.291				0.088
75	54	1.00	1.00	119	49	1.00	1.00
78	54	1.04	0.89 (0.51-1.57)	109	58	1.22	1.17 (0.68-2.01)
81	52	0.95	0.80 (0.45-1.41)	106	61	1.57	1.63 (0.94-2.83)
			0.434				0.080

We examined the variability of RA measures obtained ~4 months apart. Levels of serum RA isomers were relatively stable within women, with ratios of between-person to within-person variability all >1.0. This stability is similar to the results published by Tang et al. (23), demonstrating no change in 13-*cis*-RA and all-*trans*-RA over a 3-month period in healthy controls (n = 13). Overall, Soderlund et al. (16) reported within-person coefficients of variability of 13.4% for 13-*cis*-RA and 13.6% for all-*trans*-RA, which were not influenced by period of menstrual cycle among 17 women. The ratios of between-person to within-person variability for 13-*cis*-RA and all-*trans*-RA (1.57 and 1.17, respectively) are very similar to those we are reporting in this study (16). Yamakoshi et al. (20) also found low between-day variances in serum RA isomers, all-*trans*-RA (coefficient of variation, 11%) and 13-*cis*-RA (coefficient of variation, 8.4%), in six healthy subjects. Altogether, these results indicate that a single RA measurement may be sufficient to accurately classify a woman's RA level.

Soderlund et al. (16) have reported individuals' seasonal variation for 13-*cis*-RA, with lower mean values during winter and spring (November-March) compared with summer months; however, these differences were small and were interpreted as being of little biological significance. In these Brazilian women, we found lower median values of 13-*cis*-RA in summer, and in the multivariate analyses, women were significantly more likely to have lower 13-*cis*-RA levels in spring and summer than in fall. In contrast to Soderlund et al. (16), we found significant seasonal differences in all-*trans*-RA, with women more likely to have lower levels in winter and spring than in fall. It may be that the seasonal differences we find may be due to differences in dietary intake; however, we did not formally test this question. Future research in this area needs to be conducted.

In this study, serum RA levels were positively associated with serum retinol and β-carotene levels. Our data are consistent with a previous study in patients with inflammation, which reported lower levels of serum retinol occurring together with low serum levels of RA (19). β-Carotene supplementation (30 mg/d) was shown to increase serum levels of all-*trans*-RA after 3 months in patients with colon polyps (increase from 2.32 ± 0.26 to 3.00 ± 0.41 ng/mL); however, no increase in 13-*cis*-RA, total RA, or retinol with supplementation was observed (23). Our results are further supported by a recent finding that supplementation with retinyl palmitate substantially increased endogenous RA levels, with all-*trans*-RA having the highest percentage increase over time followed by 13-*cis*-RA (36).

The current study was conducted within a sample of women living in São Paulo between 1993 and 1996. This population of women may have different dietary practices and sociodemographic profiles than typically found among women residing in western Europe and the United States. Results from the current study are therefore applicable to countries of similar sociodemographic characteristics. Caution should be exercised in generalizing the results to populations such as western European and American women.

To address whether similar associations might be observed among U.S. women, we compared the median retinol value of our Brazilian population with that reported by the Third National Health and Nutrition Examination Survey for women ages ≥19 years to determine how different our two populations are with respect to retinoid status. As reported by Ballew et al. (35) from the Third National Health and Nutrition Examination Survey, the median serum retinol levels for women ages 19 to 30 and 31 to 50 years were 0.53 and 0.43 μg/mL, respectively. This is similar to what we observed among our Brazilian study population where values of 0.43 and 0.48 μg/mL were seen, respectively. Although Brazilian women seem to have slightly lower median retinol values than U.S. women, these differences are not as dramatic as one might expect. We also conducted analyses restricting our population to women who have serum retinol values at or above the median reported in the National Health and Nutrition Examination Survey. Using this approach, we find consistent positive associations between serum RA isomers and serum β-carotene and β-cryptoxanthin concentrations among women with serum retinol >0.43 μg/mL. These results lead us to hypothesize that we would observe similar associations in a population of U.S. women. However, these preliminary associations need to be confirmed by conducting the appropriate study within the target U.S. population.

In this study, we are reporting serum levels of 9-*cis*-RA ranging from below detection (0.3 ng/mL) up to 3.8 ng/mL and contributing a relatively high proportion of total RA. The most likely explanations for the elevated 9-*cis*-RA levels include (*a*) incomplete resolution of 9-*cis*-RA from components in the matrix or other isomers (e.g., 9,13-*dicis*-RA), (*b*) isomerization to 9-*cis*-RA during long-term storage at −20°C, and/or (*c*) unidentified dietary or environmental factors leading to elevated levels. Currently, we do not have a standard for 9,13-*dicis*-RA to compare the retention time to determine if there was coelution to address the first possible explanation. We did not see significant isomerization when spiked samples were carried through our extraction and do not believe that artifacts were created during our extraction process. However, because the serum samples used in this study were not stored under ideal conditions at −80°C, the second explanation is a possibility.

Lanvers et al. (24) reported no degradation of all-*trans*-RA or 13-*cis*-RA in plasma during storage for 3 months at −20°C, whereas others have reported long-term storage may result in degradation of 13-*cis*-RA (18). To address this issue, we examined mean levels of RA among women enrolled 2 years apart and found that the mean RA levels were similar or elevated among samples obtained during the first year of enrollment compared with those obtained during the third year (data not shown). Therefore, our RA concentrations do not seem to be affected by serum storage conditions. In addition, we have comparable overall levels of total RA to other studies (16-19), and we have compared our study to one whose samples were stored ideally (36), and there were no significant differences (data not shown). Further research in this area needs to be conducted to clarify these results.

As with all observational studies, this study has limitations that need to be recognized. The serum samples obtained within this cohort study were not originally designated for measurement of RA; therefore, not all precautions were taken to ideally store these samples, including storage at −20°C and exposure to some UV light. Similar to other biological markers, the values of RA presented in this report may not reflect the absolute value of endogenous RA due to losses that may have occurred during storage or in the extraction process. However, the relative levels of each isomer should not have changed; therefore, the associations found in this study are assumed valid. The magnitude of the associations will potentially be lower than the true association due to any methodologic errors. Serum samples were not fasting samples; therefore, there is a potential for recent food consumption to have influenced serum RA values. However, due to the low within-person variability over a ~4-month period and the small variance surrounding mean RA at each visit, we do not believe this to be an issue. As this study included Brazilian women only, our results cannot be generalized to men. We have no reason to believe that this relatively disease-free sample of women are different than other groups of women, leading us to infer that the results presented here may be of relevance to other female populations.

The Ludwig-McGill Cohort offered a unique opportunity to assess the association between endogenous RA levels and lifestyle, demographic, and nutritional factors in a sample of >500 women. There were multiple serum samples available for measurement of RA allowing for the first assessment of RA variability over time. In addition, we assessed serum retinol and carotenoids at four time points, which reduces the inherent variability of these measures, thereby reducing misclassification of serum nutrient level. Nutrient intake was estimated using Brazil-specific carotenoid food content to increase accuracy in estimating carotenoid intake (26).

RA has been given to patient populations to regress preneoplastic lesions; however, when RA is given p.o., there are significant toxicities associated. These toxicities have decreased its utilization in some populations, specifically among reproductive age women. By identifying factors that are associated with increased endogenous levels of RA, there is potential that modification of these factors will increase levels of RA, leading to prevention of preneoplastic lesions without the toxicity of supplemental treatment. This could be potentially beneficial to women of reproductive age, especially women at risk for cervical cancer, for which RA has been effective at regressing cervical dysplasia (12). Future studies need to investigate whether increased levels of endogenous RA are associated with a decreased risk of preneoplastic cervical lesions.

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