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Diet and serum micronutrients in relation to cervical neoplasia and cancer among low-income Brazilian women

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Cervical cancer is a leading cancer among women in developing countries. Infection with oncogenic human papillomavirus (HPV) types has been recognized as a necessary cause of this disease. Serum carotenoids and tocopherols have also been associated with risk for cervical neoplasia, but results from previous studies were not consistent. We evaluated the association of serum total carotene and tocopherols, and dietary intakes with the risk of newly diagnosed, histologically confirmed cervical intraepithelial neoplasia (CIN) grades 1, 2, 3 and invasive cancer in a hospital-based case-control study in São Paulo, Brazil. The investigation included 453 controls and 4 groups of cases (CIN1, $n = 140$; CIN2, $n = 126$; CIN3, $n = 231$; invasive cancer, $n = 108$) recruited from two major public clinics between 2003 and 2005. Increasing concentrations of serum lycopene were negatively associated with CIN1, CIN3 and cancer, with odds ratios (OR) (95% CI) for the highest compared to the lowest tertile of 0.53 (0.27–1.00, p for trend = 0.05), 0.48 (0.22–1.04, p for trend = 0.05) and 0.18 (0.06–0.52, p for trend = 0.002), respectively, after adjusting for confounding variables and HPV status. Increasing concentrations of serum α- and γ-tocopherols, and higher dietary intakes of dark green and deep yellow vegetables/fruit were associated with nearly 50% decreased risk of CIN3. These results support the evidence that a healthy and balanced diet leading to provide high serum levels of antioxidants may reduce cervical neoplasia risk in low-income women.

Cervical cancer is the second most common cancer among women worldwide. Disease occurrence varies widely by geographic region, with the highest rates in Latin American countries and the Caribbean, sub-Sahara Africa and parts of Asia.[1] Infection with oncogenic human papillomavirus (HPV) types is considered a necessary cause in the development of cervical cancer, and cofactors are tobacco smoking, long-term oral contraceptive use and high parity.[2–7]

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The aforementioned associations notwithstanding, the results on the relationship of nutritional factors to cervical neoplasia have not been consistent across different studies. Only a few investigations have used large numbers of subjects but were conducted mostly in developed countries. An important limitation is the absence of valid information on HPV status and distribution of types, which varies by country.[8] Thus, it has been suggested that most of the observations to date must be accepted only tentatively.[9] The study of the relationship of nutritional factors with cervical neoplasia is complicated by its multifactorial etiology, with many of the identified risk factors being correlated with nutritional and socioeconomic status.[10] Dietary components may be involved in the etiology of this disease since socioeconomic differences in risk persist after adjustment for known factors which suggests that dietary inadequacy consequent to poverty may help explain the high incidence rates in developing countries.[11]

Previous case-control studies carried out in developed countries examined the association of carotenoids (α-carotene, β-carotene, lycopene, lutein, cryptoxanthin), tocopherols and retinol in both cervical cancer and preneoplastic lesions. Although substantial evidence suggests a role for nutritional factors in the persistence or clearance of HPV infection and

the development of cervical cancer, most studies either did not include valid information on habitual diet or did not examine associations across the spectrum of preinvasive lesion grades and cervical cancer.[12–18]

The objective of the present case-control study was to examine associations between serum total carotene and tocopherols as well as dietary intakes on cervical intraepithelial neoplasia (CIN) and invasive cancer risks while controlling for HPV status.

Material and Methods

Study population

This case-control study included women attending 2 major hospitals [Instituto Brasileiro de Controle do Câncer (IBCC) and Hospital Perola Byington (HPB)] in São Paulo, Brazil, for cervical cytological screening between 2003 and 2005. IBCC and HPB are important public hospitals in the state of São Paulo: the former for cancer screening and treatment, and the latter as women's tertiary care center. We aimed to recruit prospectively newly diagnosed cases of CIN and invasive cancer. Eligible women were residents of São Paulo aged 21–65 years and had no prior hysterectomy, previous treatment for cervical neoplasia or cancer history. Women who were positive for Human Immunodeficiency Virus (HIV) or who had been pregnant or breastfeeding within 6 months of enrollment were ineligible. Patients who had been in radiotherapy or chemotherapy treatment were excluded because of the concern that cancer treatment could have affected the nutrients under investigation.

Cases included women with a histological diagnosis of CIN grades 1, 2 or 3 or invasive adenocarcinoma, adenosquamous or squamous cell carcinoma of the cervix, reviewed by 2 pathologists. During the same period, control women were selected from among those attending routine cervical screening or those referred for cytology confirmation at the same clinics where cases were diagnosed. All eligible women with a cytological diagnosis within normal limits were invited to participate.

Ethical approval was obtained from the Institutional Review Boards of the School of Public Health (University of São Paulo) and the Medical Ethical Committees of participating hospitals, and all participants provided written informed consent.

Data collection

Participants underwent a personal interview by trained dietitians blinded to the group assignment. A standardized questionnaire was used to elicit information on socioeconomic and demographic characteristics, food intake, physical activity (including both occupational and leisure activities, expressed in metabolic equivalent tasks in hour per week), smoking (individuals who had quit smoking at least 1 year before the interview were considered former smoker) and alcohol consumption, reproductive and sexual history, and other risk factors for cervical neoplasia. Self-reported ethnicity was defined in 5 categories used by the *Instituto Brasileiro de Geografia e Estatística*: white, black, oriental, mulatto or Indian.

Body mass index (BMI) was calculated (kg/m^2) based on weight and height measurements with subjects wearing light clothes and no shoes, according to standard protocols and cut-offs proposed by the World Health Organization.[19]

Food consumption was assessed using a food frequency questionnaire (FFQ) previously validated[20,21] and adapted to epidemiological studies on diet and prevention of chronic diseases in São Paulo.[22] Women were asked about usual frequency of consumption of 76 foods items and their portion sizes, an open-ended food section, and vitamin and mineral supplements during the previous year. Nutrient and food groups were analyzed using the Dietsys software version 4.01.[23] The nutrient database was based primarily on the US Department of Agriculture publications supplied by Dietsys and supplemented by the Brazilian Standard Food Composition table. Questionnaires were excluded from further analyses if energy intake was implausible (<700 or >6,000 Kcal/day), corresponding to the percentiles <2.5 or >97.5, respectively). For the present study, we investigated macronutrients, vitamins, minerals and 5 food groups: (*i*) Dark green and deep yellow vegetables and fruit (green salad, kale, broccoli, spinach, pumpkin, carrot, sweet potatoes, papaya and mango); (*ii*) total fruit and juices; (*iii*) only citrus fruit and juices; (*iv*) total vegetables; (*v*) total fruit and vegetables.

Serum micronutrient analyses

All participants were scheduled to provide a fasting blood sample within one-week after recruitment in the study. Whole blood was taken protected from light, centrifuged within 1 hr of collection and frozen at $-70^{\circ}C$ until being analyzed. Serum samples were analyzed for total carotene (α-, β- and γ-carotene), lycopene, α- and γ- tocopherols and retinol by reversed-phase high-performance liquid chromatography (HP-1100 HPLC system, Hewlett Packard, Palo Alto, CA), as previously described.[24] Peaks for total carotene that were under the quantification limits were set to zero. There were 3 samples for total carotene, 1 for lycopene and 2 for γ-tocopherol that were below the limit of quantification (detectable level of total carotene, lycopene and γ-tocopherol were respectively: 0.5, 0.2 and 0.2 μmol/l). Total serum cholesterol was measured enzymatically using an automatic device (ADVIA 1650, Bayer, East Walpole, MA). All samples were analyzed within 6 months after collection. The laboratory assayed internal and external blinded quality control specimens in every run. From the control specimens, the accuracy and interassay coefficients of variation for these analytes were within 8%.

HPV testing

Exfoliated cervical cells were collected from each woman using the DNA-Cytoliq® (Digene Brazil, São Paulo, Brazil) liquid-based system. The ectocervical and endocervical samples were collected using the brush provided with the kits

and immersed in Universal Collecting Medium™ vials. The cervical specimen was stored at 4°C until processing for cytology and HPV testing.

Laboratory personnel were blinded to the case-control status of the participants. Cervical specimens were centrifuged at 5,000 rpm for 10 min at 22–24°C. Cells were digested with 0.2 mg/ml proteinase K in 50 µl 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA buffer pH 8.0, 0.5% SDS between 2 and 16 hr at 60°C and purified. Extracted DNA was tested for presence of human DNA using a polymerase chain reaction (PCR) protocol for β-globin and for HPV DNA by a PCR protocol amplifying a highly conserved 450-bp segment in the L1 viral gene (flanked by primers MY09/11). Typing of amplified products was performed by hybridization with individual oligonucleotide probes specific for 27 HPV genital types 6/11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 56, 57, 58, 59, 66, 68, 73, 82, 83 and 84. The PCR amplification products were further tested by restriction fragment-length polymorphism (RFLP) analysis of the L1 fragment, to resolve dubious results from the dot-blot hybridization and to distinguish among HPV types that could not be identified by dot-blot hybridization with the specific probes. This allowed extending the detection range to >40 genital HPV types, including HPV types 32, 34, 62, 67, 69–72 and 89. Amplified products that hybridized with the generic probe but not with any of the type-specific probes and that also did not produce a recognizable band pattern in the RFLP analysis were considered to be positive for HPV of unknown types.

A cervical exfoliated sample was not available for 43 of 108 (39.8%) invasive cancer cases (the clinical stage distribution according to the International Federation of Gynecology and Obstetrics classification among these cases was as follows: 46%, 14 and 40% for stage I, II and III, respectively). HPV testing in such cases was done in paraffin-embedded biopsies. Cellular material was extracted from paraffin sections in 200–400 µl buffer (50 mM Tris-HCl pH 7.5; 1 mM de EDTA; 0.5% de Triton X-100) in a microwave oven set at the highest power no more than 15 sec for 4 times, followed by centrifugation at 12,000 rpm during 15 min. The sample was then incubated, centrifuged and treated to extract DNA as earlier. The quality of DNA extracts was ascertained by testing for human β-globin by PCR. Specimens were tested more than once until a definitive result was obtained. Separate assays were performed with a diluted sample in case the first amplification resulted negative for β-globin. Paraffin-embedded samples were tested by the GP5+/6+ general primer system which amplifies a conserved 150-bp segment in the L1 viral gene. Typing of amplified products was performed as described earlier.

Definition of variables

Serum concentrations of total carotene, tocopherols and retinol, dietary nutrients, food groups and other continuous variables, except age, were categorized into tertiles or quartiles based on their distribution among controls. Ethnicity was categorized as white and non-White (black women and mulatto). HPV types were classified as having either high-risk oncogenic potential or low-risk oncogenic potential. High-risk types included HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82.[25] HPV status was classified as per the following 4 hierarchical mutually exclusive categories: (*i*) HPV negative, (*ii*) only positive for low-risk HPV types, (*iii*) positive for at least 1 high-risk HPV type except HPV-16 and HPV-18 and (*iv*) positive for HPV-16 and/or HPV-18.

Statistical analysis

Median values and interquartile (IQ) ranges were calculated for serum micronutrients and dietary intake according to outcome status. Pearson χ^2 test was used to examine differences in proportions and the Mann-Whitney test for differences in continuous variables between groups. Serum tocopherol measurements were adjusted for total serum cholesterol, and dietary nutrient intakes were adjusted for total energy by the residual method.[26] Separate unconditional logistic regression models were used to calculate odds ratios (OR) and 95% confidence intervals for each of 4 outcomes (CIN 1, CIN2, CIN3 and invasive cancer) with individuals in the lowest tertile or quartile category of the exposure of interest (serum tocopherols, total carotene, foods or nutrients) as the referent group. Tests for linear trend were calculated by assigning the median value for each exposure category and modeling the values as a continuous variable. All analyses were adjusted for age, hospital, ethnicity, education and potential confounders (smoking, sexual debut, lifetime sexual partner and parity) or mediators (HPV status) if their inclusion in any of the models caused a change in the OR estimate of 10% or more. Analyses were done in STATA 9.0 (Statacorp, College Station, TX).

Results

Figure 1 shows the recruitment of participants. A total of 1,676 women were contacted between March 2003 and May 2005. Among them, 1,447 women completed the study protocol (86.3%) with 53 refusals (4.5%; 34 potential cases and 19 potential controls). Among those enrolled, 1,179 women were eligible for the present study. Overall, 121 participants were excluded for the present analysis: 64 cases of cervical neoplasia were not confirmed on biopsy, 48 were diagnosed with atypical squamous/glandular cells of undetermined significance (ASCUS or AGCUS), and 9 had negative β-globin exfoliated cervical cells for HPV testing. In total, 453 controls and four case groups were considered: 140 women diagnosed with CIN1, 126 with CIN2, 231 with CIN3 and 108 patients with cervical cancer (of them, 9 were invasive adenocarcinoma). Overall, 461 cases and 331 controls provided fasting blood specimen (76% and 73% compliance among cases and controls, respectively).

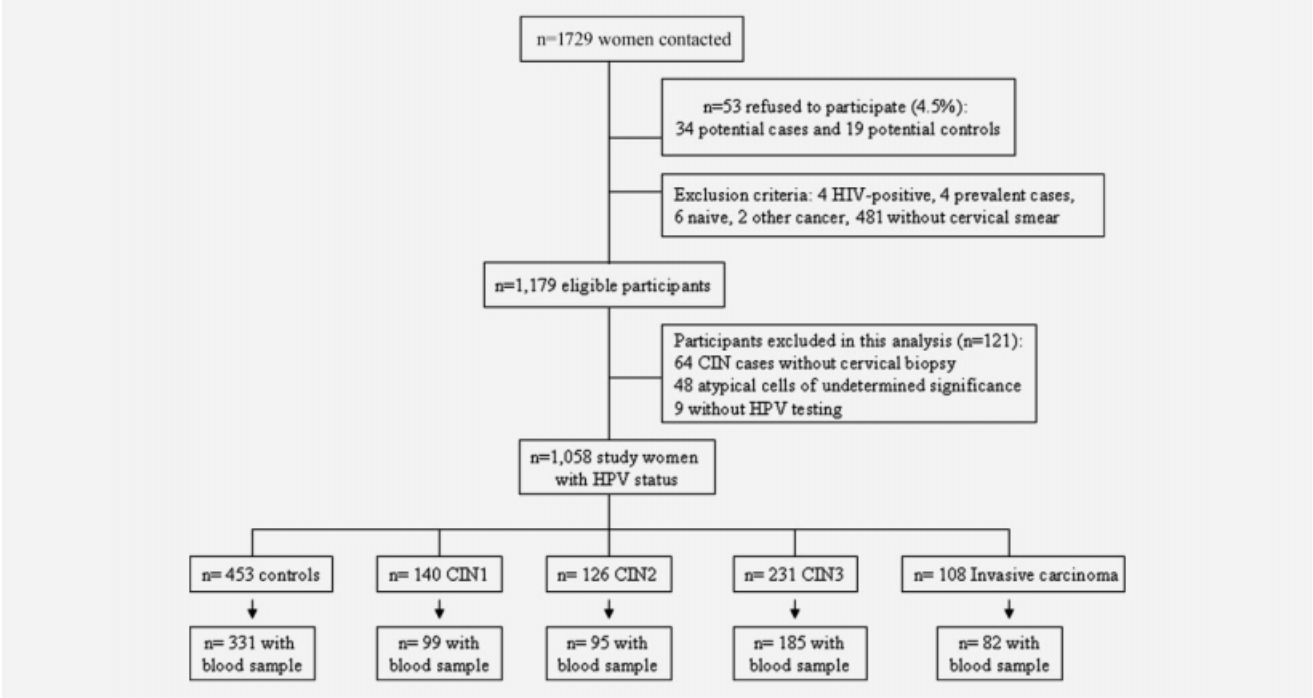

Figure 1. Flow chart with details of selection of study participants (2003–2005).

The mean ages (SD) were 40.3 (11.4), 35.2 (9.8), 35.1 (10.1), 38.1 (9.7), 46.2 (9.9) years for controls, CIN1, CIN2, CIN3 and invasive cancer cases, respectively. Most participants were non-White (68.2%) with a median monthly income (interquartile ranges, IQ) of USD 58.0 (33.5–105.0). CIN3 and cancer case groups were more likely to be older, current smokers, less educated, had lower income, had an earlier sexual debut, a higher number of sexual partners, and higher parity (Table 1). Median values (IQ) of number of lifetime Pap tests were 9 (3.3–17.0), 5 (3.0–12.0), 6 (2.0–12.5), 4 (2.0–11.0) and 5 (2.0–13.0) for controls, CIN1, CIN2, CIN3 and invasive cancer cases, respectively. HPV positivity, mainly HPV16 and/or HPV18, was higher in cases than in controls. Among cases of invasive cancer, positivity for HPV in paraffin-embedded biopsies was 76.7% ($n = 43$) and in exfoliated cervical cells was 89.2% ($n = 65$) (χ^2 test, $p = 0.081$).

Table 2 shows the concentrations of serum micronutrients and dietary intake for all groups. Missing values for dietary information were due to 2 participants without dietary data and 5 women with implausible values. Total energy and food intake were similar across study groups, except for total fruit and juices and dark green and deep yellow vegetables and fruit which were lower among CIN1 and CIN3 cases relative to controls and other case groups. Serum concentrations of total carotene and lycopene were lower for all case groups, and serum tocopherols were lower among CIN cases than among controls and cancer cases.

Any dietary and serum biomarker was associated with the lowest CIN grade, except for serum lycopene. Higher serum concentrations of lycopene were negatively associated with CIN1. In crude analysis, the OR (95%CI) for the highest compared to the lowest tertile was 0.52 (0.29–0.94, p for trend = 0.05). After adjusting for confounding variables (age, hospital, ethnicity, schooling, smoking, sexual debut, lifetime number of sexual partner and parity) the estimated relative risk was 0.56 (0.31–1.03, p for trend = 0.06). After adjusting for covariates and HPV status, the OR (95%CI) was 0.64 (0.34–1.21) for the second and 0.53 (0.27–1.00, p for trend = 0.05) for the highest tertile when compared to the lowest (data not shown).

Table 3 presents results from three multiple logistic regression models for the association between serum micronutrients and dietary intake and CIN2. In crude analysis, increasing tertiles of serum α-tocopherol and total carotene were associated with a decreasing risk of CIN2. After multiple adjustments, a trend for decreasing risk of CIN2 with increasing tertiles was noted for serum total carotene.

Table 4 presents results for the association between serum micronutrients and CIN3. Increasing serum concentrations of α- and γ-tocopherols were inversely associated with CIN3 risk; the adjusted OR for the highest compared to the lowest quartile of α-tocopherol was 0.36 (95%CI, 0.18–0.74; p for trend = 0.004), and for the highest versus lowest tertile of γ-tocopherol was 0.51 (95%CI, 0.28–0.91; p for trend = 0.02) after adjusting for confounding variables. When we additionally adjusted for HPV status, the strength of the associations was maintained for both tocopherols (p for trend = 0.03). In analyses restricted to oncogenic HPV positive women (high-risk HPV including HPV16/HPV 18), serum α-tocopherol

Table 1. Characteristics of study subjects by outcome status, São Paulo (2003–2005)

Variable	Controls No. (%)	CIN1 No. (%)	CIN2 No. (%)	CIN3 No. (%)	Invasive cancer No. (%)
Age, years					
21–30	108 (23.8)	55 (39.3)	50 (39.7)	56 (24.2)	6 (5.6)
31–40	113 (25.0)	44 (31.4)	45 (35.7)	86 (37.2)	28 (25.9)
41–50	143 (31.6)	32 (22.9)	18 (14.3)	62 (26.8)	36 (33.3)
51–65	89 (19.6)[a]	9 (6.4)[a]	13 (10.3)[a]	27 (11.8)[b]	38 (35.2)[c]
Ethnicity					
White	176 (38.9)	40 (28.6)	33 (26.2)	54 (23.4)	29 (26.9)
Non-White	277 (61.1)[a]	100 (71.4)[a]	93 (73.8)[a]	177 (76.6)[a]	79 (73.1)[a]
Years of schooling					
≥11	153 (33.8)	48 (34.3)	31 (24.6)	36 (15.6)	7 (6.5)
6–10	135 (29.8)	50 (35.7)	43 (34.1)	71 (30.7)	24 (22.2)
≤5	165 (36.4)[a]	42 (30.0)[a]	52 (41.3)[a]	124 (53.7)[b]	77 (71.3)[c]
Income per person, USD/month[1]					
≥100	150 (33.1)	38 (27.1)	33 (26.2)	42 (18.2)	22 (20.4)
51–99	150 (33.1)	38 (27.1)	40 (31.7)	69 (29.9)	28 (25.9)
≤50	150 (33.1)[a]	61 (43.6)[b]	51 (40.5)[a]	115 (49.8)[b]	58 (53.7)[b]
Cigarette smoking[1]					
Never	261 (57.6)	73 (52.1)	59 (46.8)	66 (28.6)	48 (44.4)
Former, ≤5.80 pack-year	47 (10.4)	16 (11.4)	14 (11.1)	18 (7.9)	5 (4.6)
Former, ≥5.81 pack-year	46 (10.1)	10 (7.1)	8 (6.3)	28 (12.1)	14 (13.0)
Current					
≤ 4.80 pack-year	24 (5.3)	15 (10.7)	11 (8.7)	22 (9.5)	4 (3.7)
4.81–14.0 pack-year	33 (7.3)	14 (10.0)	22 (17.5)	53 (22.9)	17 (15.7)
>14 pack-year	42 (9.3)[a]	12 (8.7)[a]	11 (8.7)[a]	44 (19.0)[b]	19 (17.6)[c]
Ageat first vaginal intercourse (years)[1]					
≥21	121 (26.7)	31 (22.1)	19 (15.1)	16 (6.9)	8 (7.4)
19–20	141 (31.1)	36 (25.7)	34 (27.0)	58 (25.1)	30 (27.8)
16–18	119 (26.3)	42 (30.0)	36 (28.6)	74 (32.0)	41 (38.0)
≤15	72 (15.9)[a]	31 (22.1)[a]	35 (27.8)[a]	82 (35.5)[b]	29 (26.8)[b]
Lifetime number of sexual partners[1]					
1	191 (42.2)	40 (28.6)	28 (22.2)	39 (16.9)	24 (22.2)
2–3	181 (40.0)	67 (47.8)	61 (48.4)	105 (45.5)	31 (28.7)
≥4	80 (17.7)[a]	33 (23.6)[a]	37 (29.4)[a]	86 (37.2)[b]	53 (49.1)[a]
Number of pregnancies					
Never	57 (12.6)	16 (11.4)	13 (10.3)	10 (4.3)	4 (3.7)
1	82 (18.1)	28 (20.0)	16 (12.7)	20 (8.6)	10 (9.2)
2	111 (24.5)	30 (21.4)	33 (26.2)	24 (10.5)	18 (16.7)
3	90 (19.9)	34 (24.3)	21 (16.7)	59 (25.5)	12 (11.1)
≥4	113 (24.9)[a]	32 (22.9)[a]	43 (34.1)[a]	118 (51.1)[b]	64 (59.3)[c]
Oral contraceptive use					
Never	112 (24.7)	27 (19.3)	27 (21.4)	60 (26.0)	30 (27.8)
Former	261 (57.6)	71 (50.7)	68 (54.0)	119 (51.5)	70 (64.8)
Current	80 (17.7)[a]	42 (30.0)[a]	31 (24.6)[a]	52 (22.5)[a]	8 (7.4)[b]
HPV status					
HPV negative	324 (71.5)	47 (33.6)	20 (15.9)	15 (6.5)	17 (15.7)
Positive only for low-risk HPV	24 (5.4)	4 (2.8)	7 (5.5)	3 (1.3)	5 (4.6)
Positive for at least one high-risk type except HPV-16/HPV-18	45 (9.9)	43 (30.7)	31 (24.6)	52 (22.5)	15 (14.0)
Positive for HPV-16 and/or HPV-18	60 (13.2)[a]	46 (32.9)[a]	68 (54.0)[b]	161 (69.7)[c]	71 (65.7)[b]

[1]Totals do not coincide due to missing values for some variables. Values in the same row with different superscript letters are significantly different in percentages, $p \leq 0.05$.

Table 2. Medians (interquartile ranges) for serum micronutrient concentrations and dietary intakes by outcome status (São Paulo, 2003–2005)

	Controls	CIN1 cases	CIN2 cases	CIN3 cases	Invasive cancer
Serum concentrations (μmol/l)					
n	331	99	95	185	82
Total carotene	0.80 (0.47–1.40)[a]	0.70 (0.35–1.20)[a]	0.57 (0.31–0.94)[b]	0.50 (0.29–0.95)[b]	0.46 (0.29–0.79)[b]
Lycopene	1.10 (0.60–1.60)[a]	0.89 (0.40-1.35)[b]	0.77 (0.50–1.57)[b]	0.74 (0.41–1.30)[b]	0.68 (0.35–1.09)[b]
Retinol	1.70 (1.40–2.30)[a]	1.77 (1.22–2.33)[a]	1.70 (1.11–2.30)[a]	1.72 (1.22–2.44)[a]	1.51 (1.03–1.93)[b]
α-tocopherol	16.59 (12.50–22.10)[a]	15.10(11.60–18.86)[b]	12.40 (9.75–17.99)[b]	13.74 (10.10–18.00)[b]	15.22 (11.22–11.55)[a]
γ-tocopherol	1.90 (1.50–2.40)[a]	1.68 (1.30–2.30)[b]	1.60 (1.20–2.30)[b]	1.60 (1.20–2.10)[b]	1.90 (1.30–2.83)[a]
Daily dietary intakes					
n	451	137	125	230	108
Total energy (kcal/day)	2,058 (1,658–2,586)[a]	2,109 (1,756–2,534)[a]	2,191 (1,781–2,703)[a]	2,149 (1,728–2,750)[b]	2,133 (1,538–2,630)[a]
Total fruits and juice (g/day)	161.8 (69.1–327.4)[a]	137.9 (60.4–132.8)[b]	164.9 (62.7–287.4)[a]	124.2 (44.8–225.8)[b]	134.4 (49.8–387.1)[a]
Total vegetables (g/day)	99.9 (62.9–327.4)[a]	92.7 (61.5–132.8)[a]	107.1 (66.2–162.3)[a]	97.6 (61.0–143.3)[a]	91.1 (45.3–148.2)[a]
Dark green and deep yellow vegetables and fruits (g/day)	28.2 (12.1–59.0)[a]	20.1 (7.7–46.6)[b]	27.9 (10.6–50.7)[a]	16.2 (6.7–37.6)[b]	22.8 (4.4–59.4)[a]
Alcohol (g/day)	0 (0–20.0)[a]	1.4 (0–50.5)[b]	0.7 (0–27.5)[a]	0.7 (0–35.1)[b]	0 (0–4.8)[c]

Values in the same row with different superscript letters are significantly different in medians, $p < 0.05$ (Mann Whitney U test).

concentrations were negatively associated with CIN3 [adjusted OR (95%CI): 0.44 (0.18–1.08), p for trend = 0.04] (data not shown). Serum lycopene, dietary intakes of dark green and deep yellow vegetables/fruit, and carrots were inversely associated with CIN3 [adjusted OR (95%CI): 0.49 (0.28–0.88), 0.52 (0.31–0.87) and 0.56 (0.34–0.92), respectively], when participants in the highest tertiles were compared to those in the lowest tertiles after adjusting for confounding. These associations were not considerably different after additional adjustment for HPV status (p for trend = 0.05). When the analysis was restricted to HPV16 and/or HPV18-positive women, the most oncogenic types for cervical cancer, and inverse association was observed between serum lycopene, total carotene and CIN3: adjusted OR (95%CI) for the highest compared to the lowest tertile was 0.19 (0.05–0.70; p for trend = 0.01), 0.26 (0.08–0.91; p for trend = 0.02, respectively (data not shown).

Associations between selected serum micronutrients and cervical cancer are reported in Table 5. Only increasing serum concentrations of lycopene were negatively associated with the risk of cancer after adjusting for confounding variables and HPV status. The adjusted OR (95% CI) for the highest versus the lowest tertile was 0.18 (0.06–0.52; p for trend = 0.002) for lycopene.

We conducted an additional analysis among controls to investigate factors associated with HPV positivity. Dietary intakes of dark green vegetables were inversely associated with HPV positivity [adjusted OR (95%CI): 0.51 (0.31–0.83), p for trend = 0.007] when comparing participants above versus below median values, adjusting for the same covariates (age, hospital, ethnicity, education, sexual debut, lifetime sexual partners and parity).

In an additional analysis we compared cases with different lesion grades to study potential predictors of progression. For precision, we categorized markers as either below or above the median values. A high total carotene concentration was associated with reduced odds of cancer risk when compared to CIN1 [adjusted OR (95%CI): 0.40 (0.16–0.98) and CIN3 cases [adjusted OR (95%CI): 0.43 (0.21–0.88). A high serum lycopene concentration was negatively associated with progression from CIN2 to cancer [adjusted OR (95%CI): 0.44 (0.19–0.99). For dietary intake, dark green and deep yellow vegetables and fruit reduced the risk of progression from CIN2 to CIN3: [adjusted OR (95%CI): 0.44 (0.26–0.75) (data not shown).

Discussion

In this case-control study we examined associations between several nutritional markers and different grades of cervical lesions while controlling for HPV status. Nutritional status was assessed by a validated food frequency questionnaire and biochemical measurements. Our findings suggest that (i) serum lycopene concentration was associated with reduced risk of cervical dysplasia and invasive cancer, (ii) serum tocopherols were inversely associated with CIN2 and CIN3, after

Table 3. Odds ratios (OR) and corresponding 95% confidence intervals (CI) among cases of CIN2 and controls according to tertiles of serum micronutrients and dietary intakes (São Paulo, 2003–2005)

	CIN2 cases No. (%)	Controls No. (%)	OR (95% CI)		
			Model 1	Model 2	Model 3
Serum concentrations (μmol/l)					
α-tocopherol[1]					
0.18–0.61	56 (58.9)	114 (34.9)	1.00	1.00	1.00
0.61–0.82	19 (20.0)	108 (33.0)	0.38 (0.21–0.69)	0.43 (0.22–0.84)	0.39 (0.18–0.84)
0.82–1.69	20 (21.1)	105 (32.1)	0.45 (0.25–0.81)	0.61 (0.30–1.21)	0.57 (0.26–1.24)
p for trend			0.003	0.07	0.08
γ-tocopherol[1]					
0.32–0.96	45 (47.4)	112 (34.3)	1.00	1.00	1.00
0.96–1.14	17 (17.9)	109 (33.3)	0.40 (0.21–0.75)	0.40 (0.19–0.84)	0.34 (0.15–0.80)
1.14–1.82	33 (34.7)	106 (32.4)	0.87 (0.51–1.51)	1.10 (0.59–2.07)	0.92 (0.45–1.86)
p for trend			0.52	0.92	0.70
Total carotene					
0.07–0.60	51 (53.7)	119 (36.0)	1.00	1.00	1.00
0.60–1.09	25 (26.3)	101 (30.5)	0.63 (0.36–1.11)	0.82 (0.42–1.58)	0.96 (0.45–2.00)
1.09–6.23	19 (20.0)	111 (33.5)	0.45 (0.24–0.83)	0.61 (0.30–1.24)	0.70 (0.32–1.53)
p for trend			0.008	0.17	0.40
Lycopene					
0.08–0.78	49 (51.6)	107 (32.3)	1.00	1.00	1.00
0.78–1.39	18 (18.9)	106 (32.0)	0.43 (0.23–0.80)	0.51 (0.25–1.03)	0.39 (0.17–0.87)
1.39–6.30	28 (29.5)	118 (35.6)	0.63 (0.36–1.10)	0.84 (0.45–1.58)	0.74 (0.36–1.52)
Dietary intakes (g/day)			0.08	0.52	0.36
Dark green and deep yellow vegetables and fruits					
0–16.7	46 (36.8)	148 (32.8)	1.00	1.00	1.00
16.7–45.7	42 (33.6)	151 (33.5)	0.95 (0.58–1.55)	1.03 (0.60–1.79)	1.08 (0.59–1.99)
45.7–499.0	37 (29.6)	152 (33.7)	0.93 (0.56–1.54)	1.26 (0.71–2.24)	1.38 (0.72–2.64)
p for trend			0.78	0.45	0.34
Carrots					
0–69.0	46 (36.8)	152 (33.7)	1.00	1.00	1.00
69.0–203.0	44 (35.2)	148 (32.8)	1.02 (0.63–1.66)	1.29 (0.75–2.23)	1.36 (0.73–2.52)
203.0–1321.0	35 (28.0)	151 (33.5)	0.84 (0.51–1.40)	1.24 (0.70–2.21)	1.23 (0.64–2.36)
p for trend			0.52	0.44	0.50

Model 1: adjusted for age groups (21–30, 31–40, 41–50, and 51–65 years). Model 2: adjusted for age groups, hospital, ethnicity (white, non–white), schooling (≤ 5, 6–10, ≥ 11 y), smoking (never, former ≤5.80 pack-year, former ≥5.81 pack-year, current ≤4.80 pack-year, current 4.81–14.0 pack-year, current >14 pack-year), sexual debut (≥21, 19–20, 16–18, ≤15 years), life time sexual partner (1, 2–3 and ≥4 partners), and parity (0, 1, and ≥2 pregnancies). Model 3: model 2 and adjusted additionally for HPV status (negative for HPV, low-risk HPV, high-risk HPV except HPV16/HPV18, HPV16 and/or 18).
[1]Adjusted for serum cholesterol by residual method.

empirical adjustment for confounding variables and HPV status. These findings were consistent in restricted case-control analysis using only HPV-16 and/or HPV-18 positive women for serum lycopene, total carotene and CIN3.

Previous epidemiologic studies found that blood lycopene levels were inversely associated with risk of CIN1, CIN2 and CIN3 with risk reductions ranging from 40 to 72% for higher serum levels after adjusting for confounding variables and HPV.[12,15,17,26] However, only 1 study showed a significant linear trend.[17]

An important limitation of our hospital-based case-control study is the potential low efficiency in covariate adjustment because of the lack of age and ethnicity-matching in the design. We adjusted for these variables in the models, but it is difficult to ascertain that we succeeded in completely controlling for the confounding effect of these factors since age,

Table 4. Odds ratios (OR) and corresponding 95% confidence intervals (CI) among cases of CIN3 and controls according to serum micronutrients and dietary intakes (São Paulo, 2003–2005)

Exposures	CIN3 cases No. (%)	Controls No. (%)	OR (95%CI) Model 1	Model 2	Model 3
Serumconcentrations (μmol/l)					
α-tocopherol[1] (quartiles)					
0.18–0.55	84 (45.4)	84 (25.7)	1.00	1.00	1.00
0.55–0.73	41 (22.2)	90 (27.5)	0.41 (0.25–0.68)	0.47 (0.25–0.88)	0.60 (0.26–1.35)
0.73–0.90	40 (21.6)	75 (22.9)	0.49 (0.30-0.79)	0.55 (0.29–1.01)	0.52 (0.22–1.22)
0.90–1.69	20 (10.8)	78 (23.9)	0.26 (0.15–0.47)	0.36 (0.18–0.74)	0.40 (0.16–1.01)
p for trend			<0.001	0.004	0.03
γ-tocopherol[1] (tertiles)					
0.32–0.96	98 (53.0)	112 (34.3)	1.00	1.00	1.00
0.96–1.14	46 (24.8)	109 (33.3)	0.47 (0.30–0.73)	0.42 (0.23–0.75)	0.44 (0.20–0.95)
1.14–1.82	41 (22.2)	106 (32.4)	0.46 (0.29–0.73)	0.51 (0.28–0.91)	0.45 (0.21–0.97)
p for trend			<0.001	0.01	0.03
Total carotene (tertiles)					
0.07–0.60	107 (58.2)	119 (36.0)	1.00	1.00	1.00
0.60–1.09	40 (21.7)	101 (30.5)	0.47 (0.30–0.75)	0.46 (0.25–0.84)	0.48 (0.23–1.05)
1.09–6.23	37 (20.1)	111 (33.5)	0.39 (0.25–0.62)	0.53 (0.29–0.96)	0.57 (0.25–1.26)
p for trend			<000.1	0.01	0.10
Lycopene (tertiles)					
0.08–0.78	96 (51.9)	107 (32.3)	1.00	1.00	1.00
0.78–1.39	48 (25.9)	106 (32.0)	0.54 (0.35–0.85)	0.61 (0.35–1.06)	0.47 (0.22–0.99)
1.39–6.30	41 (22.2)	118 (35.7)	0.43 (0.27–0.68)	0.49 (0.28–0.88)	0.48 (0.22–1.04)
p for trend			<0.001	0.01	0.05
Dietary intakes (g/day, tertiles)					
Dark green and deep yellow vegetables and fruits					
0–16.7	118 (51.3)	148 (32.8)	1.00	1.00	1.00
16.7–45.7	68 (29.6)	151 (33.5)	0.59 (0.40–0.86)	0.66 (0.41–1.06)	0.77 (0.43–1.39)
45.7–499.0	44 (19.1)	152 (33.7)	0.39 (0.25–0.59)	0.52 (0.31–0.87)	0.52 (0.27–1.00)
p for trend			<0.001	0.01	0.05
Carrot					
0–69.0	113 (49.1)	152 (33.7)	1.00	1.00	1.00
69.0–203.0	68 (29.6)	148 (32.8)	0.64 (0.43–0.93)	0.77 (0.48–1.23)	0.87 (0.48–1.59)
203.0–1321.0	49 (21.3)	151 (33.5)	0.46 (0.31–0.70)	0.56 (0.34–0.92)	0.50 (0.27–0.95)
p for trend			<0.001	0.02	0.04

Model 1: adjusted for age groups (21–30, 31–40, 41–50, and 51–65 years). Model 2: adjusted for age groups, hospital, ethnicity (White, non-White), schooling (≤5, 6–10, ≥ 11y), smoking (never, former ≤5.80 pack-year, former ≥5.81 pack-year, current ≤4.80 pack-year, current 4.81–14.0 pack-year, current >14 pack-year), sexual debut (≥21, 19–20, 16–18, ≤15 years), lifetime sexual partner (1, 2–3 and ≥4 partners), and parity (0, 1and ≥2 pregnancies). Model 3: model 2 and adjusted additionally for HPV status (negative for HPV, low-risk HPV, high-risk HPV except HPV16/HPV18, HPV16 and/or 18).
[1]Adjusted for serum cholesterol by residual method.

ethnicity, socio-economic status, high parity, smoking, long-term oral contraceptive use are correlated with nutritional status.[10,11] Another concern is related to the study design, which does not permit assessing whether the identified relations resulted from a direct causal influence of nutritional status on risk or because of systemic effects that the disease had on the patients possibly leading to loss of nutrients. This possible effect may seem to be suggested in Table 2 as serum concentrations decrease by disease severity for total carotene, lycopene and α-tocopherol (*p* for trend < 0,001). However, the

Table 5. Odds ratios (OR) and corresponding 95% confidence intervals (CI) among cases of invasive cervical cancer and controls according to tertiles of serum micronutrientsand dietary intakes (São Paulo, 2003–2005)

	Cancer cases No. (%)	Controls No. (%)	OR (95%CI)		
			Model 1	Model 2	Model 3
Serum concentrations (μmol/l)					
α-tocopherol[1]					
0.18–0.61	35 (43.8)	114 (34.9)	1.00	1.00	1.00
0.61–0.82	19 (23.8)	108 (33.0)	0.54 (0.29–1.00)	0.89 (0.40–1.99)	0.67 (0.25–1.74)
0.82–1.69	26 (32.5)	105 (32.1)	0.70 (0.39–1.27)	1.74 (0.79–3.86)	1.49 (0.59–3.74)
p for trend			0.22	0.20	0.48
Lycopene					
0.08–0.78	48 (58.5)	107 (32.3)	1.00	1.00	1.00
0.78–1.39	23 (28.1)	106 (32.0)	0.42 (0.24–0.76)	0.66 (0.30–1.45)	0.56 (0.24–1.33)
1.39–6.30	11 (13.4)	118 (35.7)	0.17 (0.08–0.35)	0.37 (0.15–0.93)	0.18 (0.06–0.52)
p for trend			<0.001	0.03	0.002
Total carotene					
0.07–0.60	53 (64.6)	119 (36.0)	1.00	1.00	1.00
0.60–1.09	18 (22.0)	101 (30.5)	0.34 (0.18–0.63)	0.46 (0.21–1.03)	0.45 (0.18–1.18)
1.09–6.23	11 (13.4)	111 (33.5)	0.19 (0.09–0.38)	0.52 (0.22–1.23)	0.46 (0.17–1.24)
p for trend			<0.001	0.06	0.08
Dietary intakes (g/day)					
Dark green and deep yellow vegetables and fruits					
0–16.7	48 (44.4)	148 (32.8)	1.00	1.00	1.00
16.7–45.7	26 (24.1)	151 (33.5)	0.51 (0.30–0.88)	0.74 (0.34–1.48)	0.85 (0.39–1.85)
45.7–499.0	34 (31.5)	152 (33.7)	0.62 (0.37–1.00)	1.23 (0.63–2.42)	1.55 (0.73–3.29)
p for trend			0.06	0.58	0.27

Model 1: adjusted for age groups (21–30, 31–40, 41–50, and 51–65 y). Model 2: adjusted for age groups, hospital, ethnicity (White, non-White), schooling (≤5, 6–10, ≥ 11y), smoking (never, former ≤5.80 pack-year, former ≥5.81 pack-year, current ≤4.80 pack-year, current 4.81–14.0 pack-year, current >14 pack-year), sexual debut (≥21, 19–20, 16–18, ≤15 y), lifetime sexual partner (1, 2–3 and ≥4 partners),and parity (0, 1 and ≥2 pregnancies). Model 3: model 2 and adjusted also for HPV status (negative for HPV, low-risk HPV, high-risk HPV except HPV16/HPV18, HPV16and/or 18).
[1]Adjusted for serum cholesterol by residual method.

fact that the associations we observed increased in strength across the spectrum of lesion grades suggests that a true influence of the biomarkers on carcinogenesis is more likely to be correct interpretation, since systemic effects are essentially minimal for the pre-invasive stages of cervical neoplasia.

Cohort studies that assessed clearance of oncogenic HPV infection have reported faster clearance for those in the highest versus the lowest levels of serum lycopene.[27,28] However, in the Ludwig-McGill cohort study, also conducted in São Paulo, women in the highest tertile of *cis*-lycopene (most common isomer in blood) concentrations were twice as likely than those in the lowest tertile to have a persistent oncogenic HPV infection,[29] which may have been due to the need in the latter study of using average concentrations from multiple time points including samples collected after the infection had initiated.

Epidemiologic studies have found that higher blood β-carotene concentrations were protective against all CIN grades with risk reductions of 30–54%.[15–17] In our study, total serum carotene concentration was negatively associated with progression from CIN1 to cancer, and from CIN3 to cancer after adjusting for confounding variables and HPV status. In prospective studies, clearance of transient high-risk HPV infection was strongly associated with plasma β-carotene levels.[28] Our observations are consistent with the latter; it is possible that the effect of total carotene on cervical cancer may be exerted mostly in the early stages of carcinogenesis *via* mediation of HPV infection persistence.

In previous case-control studies, higher circulating levels of α-tocopherol were associated with a 50–70% risk reduction for CIN of all grades after adjusting for age, ethnicity, income, smoking, alcohol and HPV infection.[15–16] Two other studies failed to find any association between α-tocopherol and cervical dysplasia possibly due to lack of adjustment for blood cholesterol—a strongly correlated nutrient necessary for the transport of serum vitamin E.[17–18] For blood γ-

tocopherol no significant association was found in multiple models.[13] Interestingly, however, Giuliano *et al.*[30] found that serum concentrations of α- and γ-tocopherol were significantly lower in women with persistent HPV. Comparable results for α-tocopherol but not for γ-tocopherol were found in two other studies.[28,29]

Serum carotenoids and tocopherols may influence risk of cervical neoplasia *via* antioxidant activity scavenging reactive oxygen species (ROS), thus reducing toxic effects to cell membranes, cellular proteins, and nucleic acids.[31] Polyunsaturated fatty acids in cell membranes can be oxidized by ROS, which affects the fluidity and integrity of the membrane with consequent changes in the distribution and function of cellular receptors.[32] Additional anti-inflammatory mechanisms have been reported for tocopherols, such as inhibitory protein kinase C (PKC) activity, inhibitory activity of enzymes involved in eicosanoid biosynthesis, and in inhibiting COX-2-mediated biosynthesis of PGE_2.[33]

In this investigation, we did not find any association between serum retinol and CIN or cervical cancer, in contrast to previous studies.[16–18] On the other hand, we found that increased dietary intake of dark green and deep yellow vegetables and fruit, and carrots, which are important dietary sources of α-carotene, β-carotene, β-cryptoxanthin and lutein, were negatively associated with CIN3.

There is growing evidence that high intake of foods rich in β-carotene and lutein have an important role on immune response possibly acting against persistence of HPV infection. Giuliano *et al.*[33] found a reduced risk of HPV persistence among those with a high consumption frequency of papaya and orange. In our study, intake of fat group and saturated fatty acid were positively associated with HPV infection probably reflecting participants' lifestyle since these food groups were inversely correlated with fruit intake (Spearman correlation coefficient between fat intake and fruit and fruit juice intake $= -0.17$, $p < 0.001$).

Additional limitations that apply to case-control studies should be considered when interpreting our findings. Inaccurate dietary measurement could potentially have influenced our observed associations. Random errors in dietary measures more likely would have accounted for biases towards the null, thus attenuating the magnitude of the associations and their dose-response trends. On the other hand, it has been reported that case subjects can remember and report their previous experience differently than control subjects, who are free of disease. To minimize possible information bias, we additionally analyzed serum micronutrients, such as circulating carotenoids, which has been considered a good biomarker of fruit and vegetables consumption.[34] Another potential limitation in our study is related to HPV testing in paraffin-embedded biopsies used in around 40% of cancer cases. A higher proportion of late stage disease was observed among women whose HPV status was diagnosed from biopsies (χ^2 test, $p = 0.01$). The prevalence of HPV infection was lower (although not significantly) in biopsy samples when compared to exfoliated cervical cells. The failure to detect HPV DNA in 10/43 (23.3%) may have been due to the absence of HPV DNA in the analyzed sample or disruption of PCR target sequence due to viral integration.[2] To investigate the effect of controlling for HPV status determined from different samples, we conducted restricted analyses considering only HPV status based on exfoliated cervical cells or on biopsies. The ORs for invasive cancer were statistically comparable across HPV sampling sources for serum lycopene. However, the same restricted analyses for total serum carotene indicated a significant negative trend in the association only among HPV identified using biopsies (data not shown). Although an effect from misclassification of HPV status cannot be ruled out we were reassured that, apart from a loss of precision, the directionality of the associations were maintained, irrespective of sample source.

In conclusion, in this case-control study conducted in a country with a high incidence of cervical cancer we found strong inverse associations between serum lycopene and tocopherols as well as dietary intake of dark green and deep yellow vegetables and fruit and CIN and invasive cancer risks. Our findings corroborate previous epidemiologic evidence that antioxidants status may reduce risk of cervical neoplasia. Therefore, in addition to secondary prevention by screening and treatment of CIN, a balanced diet with an emphasis on antioxidant-rich fruit and vegetables may be an important preventive component against development of cervical cancer in most populations. Future research is needed to determine if serum antioxidants are associated in the early stage of carcinogenesis, in the persistence of incident oncogenic HPV infection, CIN 1 and CIN2.

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Appendix - The Brazilian Investigation Into Nutrition and Cervical Cancer Prevention Study Team (Brinca Study)

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