

A Randomized, Double Blind, Phase III Trial Using Oral β -Carotene Supplementation for Women with High-Grade Cervical Intraepithelial Neoplasia¹

Kristin A. Keefe, Michael J. Schell, Cheryl Brewer, Michael McHale, Wendy Brewster, Julia A. Chapman, G. Scott Rose, D. Scott McMeeken, Westley Lagerberg, Yei-Mei Peng, Sharon P. Wilczynski, Hoda Anton-Culver, Frank L. Meyskens, and Michael L. Berman²

Division of Gynecologic Oncology [K. A. K., M. M., W. B., M. L. B.] and Chao Family Comprehensive Cancer Center [K. A. K., M. J. S., C. B., M. M., W. B., J. A. C., G. S. R., D. S. M., W. L., H. A.-C., F. L. M., M. L. B.], University of California, Irvine, Medical Center, Orange, California 92868; Epidemiology Division, Department of Medicine, University of California, Irvine, Irvine, California 92697 [W. B., H. A.-C.]; Division of Gynecologic Oncology, Women and Infant's Hospital, Brown University, Providence, RI 02905 [K. A. K.]; Department of Biostatistics, University of North Carolina, Chapel Hill, North Carolina 27514 [M. J. S.]; Division of Gynecologic Oncology, University of Illinois at Chicago, Peoria, Illinois [C. B.]; Division of Gynecologic Oncology, Bethesda Naval Hospital, San Diego, California 92134 [M. M.]; Division of Gynecologic Oncology, University of Kansas Medical Center, Kansas City, Kansas 66101 [J. A. C.]; Division of Gynecologic Oncology, Walter Reed Army Medical Center, Washington, DC 20012 [G. S. R.]; Division of Gynecologic Oncology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73101 [S. S. M.]; Leon Levy Cancer Center, University of Arizona, Tucson, Arizona 85701 [Y.-M. P.]; and City of Hope Medical Center, Duarte, California 91009 [S. P. W.]

Abstract

To evaluate the effect of daily β -carotene (30 mg) versus placebo over a 2-year period on cervical intraepithelial neoplasia (CIN) 2 and 3 lesions. Human papillomavirus (HPV) typing was done to determine whether lesion regression was related to HPV. Micronutrient levels were measured to determine whether levels were predictive of regression. Variables that influence the risk of HPV infection and CIN, such as cigarette smoking and sexual behavior, were evaluated. Women were randomized to β -carotene or placebo, with cytology and colposcopy every 3 months. Cervical biopsies were performed before treatment and after 6 and 24 months to evaluate response. Persistence of or progression to CIN 3 resulted in removal from the study, whereas treatment continued for 2 years on all others. The presence and type of HPV was determined by PCR. Response was defined as an improvement in CIN by 2 grades. Mantel-Haenszel χ^2 test was used to analyze response to treatment. Fisher's

exact test was used to determine the effect of HPV and CIN grade on response. Wilcoxon's rank-sum tests were used to compare micronutrient levels between groups. Twenty-one of 124 enrolled women were not randomized because they either moved, became pregnant, voluntarily withdrew, or the pathological review of their initial cervical biopsies did not confirm CIN 2 or 3. Of the remaining 103 women, 33 experienced lesion regression, 45 had persistent or progressive disease, and 25 women did not complete the study and were considered nonresponders in the final analysis. The overall regression rate (32%) was similar between treatment arms and when stratified for CIN grade. Data on 99 women with HPV typing showed that 77% were HPV-positive and 23% HPV-negative at enrollment. HPV-positive lesions were subdivided into indeterminate-, low-, and high-risk categories; the response rate was highest for women with no HPV detected (61%), lower for indeterminate/low-risk (30%), and lowest for high-risk (18%; $P = .001$). CIN regression was negatively correlated with retinol levels. In conclusion, β -carotene does not enhance the regression of high-grade CIN, especially in HPV-positive subjects.

Introduction

Numerous observational studies have demonstrated an inverse relationship between the consumption of diets rich in fruits and vegetables and the risk of developing preinvasive neoplasia or cancer at a number of organ sites, including the cervix (1–4). Case-control studies exploring the relationship between diet and cervical dysplasia have demonstrated that a low intake of vitamins A, C, and β -carotene was associated with an increased risk of cervical dysplasia (5, 6). Measurement of plasma levels of various micronutrients has supported this observation, with low levels typically associated with an increased risk of CIN³ and cervical cancer. For example, Palan *et al.* reported lower levels of vitamin C and β -carotene in the plasma of women with cervical dysplasia than in normal controls (1, 7). A similar finding was reported by Batieha *et al.* (8) in a nested case control study in which 50 women who developed cervical cancer or carcinoma *in situ* over a 15-year period had significantly lower prediagnostic serum β -carotene levels than 99 matched controls (odds ratio, 3.1; 95% confidence interval, 1.2–8.1).

Although the primary mechanism of action of β -carotene remains elusive, its chemoprotective effect might be mediated

Received 4/6/01; revised 7/19/01; accepted 7/26/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by National Cancer Institute RO1-CA-50364. Presented at the Society of Gynecologic Oncology, 30th Annual Meeting, February 6, 2000, San Diego, California.

² To whom requests for reprints should be addressed, at University of California, Irvine, Medical Center, 101 The City Drive South, Building 23, Room 107, Orange, California, 92868. Phone: (714) 456-6570; Fax: (714) 456-6463.

³ The abbreviations used are: CIN, cervical intraepithelial neoplasia; OCP, oral contraceptive; Pap smear, Papanicolaou smear; UCI, University of California, Irvine, Medical Center; QC/QA, quality control/quality assurance.

through its effect on epithelial cell proliferation and differentiation. For example, retinoids inhibit HPV 16-induced transformation in keratinocytes and decrease the expression of E6 and E7 HPV oncoproteins, thereby interfering with uncontrolled cell growth. In addition, β -carotene itself has been shown to inhibit tumor cell proliferation by reducing epidermal growth factor receptor levels on keratinocytes that have been immortalized with HPV 16 (9).

Given the promise of diet and micronutrient supplementation on cancer chemoprevention in numerous epidemiological trials, four randomized clinical trials using β -carotene as a possible chemopreventative agent for cervical cancer have been completed to date with mixed results (10–13). These trials have evaluated the relationship between β -carotene supplementation and the regression of CIN; the relationship of serum and tissue micronutrient levels on the regression rate of CIN; and the role of β -carotene in modulating various intermediate biomarkers of cervical cancer risk, such as causing HPV to disappear from cervical tissue and CIN to regress. In addition to these controlled clinical trials using β -carotene given *p.o.*, *trans*-retinoic acid has also been applied topically to the cervixes of women with biopsy-proven CIN 2 and 3 (14). This agent has been shown to lead to regression of CIN 2, but not CIN 3, lesions.

This report presents data from a 2-year, randomized, placebo-controlled, chemoprevention trial evaluating the effect of β -carotene in the treatment of high-grade CIN (CIN 2 and 3). Intermediate biomarkers of cervical cancer risk, including grade of CIN and HPV presence, and risk category (high, intermediate, low, indeterminate, or none) were also evaluated. Serum and vaginal micronutrient levels were measured to determine whether they were predictive of lesion regression. Finally, the influence of other important epidemiological variables, such as smoking, OCP use, and number of pregnancies and sexual partners, was investigated.

Patients and Methods

Women at least 18 years of age with biopsy-proven CIN 2 or 3 within 3 months of enrollment into the study were recruited from the UCI patient population and from nearby clinics and colleges through printed notices and newspaper and radio advertisements. Nine hundred and eighty-two patients were screened for eligibility, and the patients were referred to the study from the above sources as follows: 47% from the UCI Pathology Department, 13% from UCI clinics, 2% from nearby universities, 12% from private physicians, 9% by self-referral, 13% through advertisements, and 3% from other sources. Respondents with CIN 2 or CIN 3 on ectocervical biopsy before the initial visit were eligible for enrollment into the study. Enrollment criteria also included the following: agreement by two pathologists on the cervical biopsy histology, colposcopic evaluation demonstrating a persistent lesion compatible with CIN 2 or 3 at least 1 month after the initial biopsy, a negative ECC (endocervical curetage), and no suspicion of cancer on cytology. Women were allowed to consume a maximum of two multivitamins/day. Subjects were stratified for CIN grade before randomization. After giving written informed consent, according to UCI Institutional Review Board guidelines, subjects began a 1-month run-in period, taking placebo pills to evaluate compliance. Subjects who consumed at least 75% of the pills during that time were considered compliant and were randomized to receive daily doses of either 30 mg of β -carotene or a placebo (Hoffman La Roche, Princeton, NJ). Women were reimbursed a total of \$100.00 for participating in this study; \$50.00 at randomization and \$50.00 at completion of the study.

Compliance after enrollment was measured as the percentage of pills taken between visits. Subjects who took <75% of pills after randomization were considered noncompliant and were taken off study, but they were included in the intention-to-treat analysis at study completion. Toxicity was evaluated every 3 months, based on Southwestern Oncology Group criteria, by asking patients to complete a questionnaire that assessed skin yellowing, nausea, vomiting, diarrhea, anorexia, and fatigue.

Response Criteria. The 2-year protocol required a Pap smear and colposcopic examination to be performed every 3 months as well as directed cervical biopsies after 6 and 24 months by physicians in the Division of Gynecological Oncology at the University of California, Irvine, under the direction of the principal investigator. Cervicography was also performed at enrollment and after 6 and 24 months and compared with the physician's colposcopic exam and the pathological findings (National Testing Labs, St. Louis, MO). All health care providers involved in the study were blinded to the patient's treatment arm. Additional biopsies were performed if the colposcopic examination or Pap smear suggested disease progression during any 3-month follow-up examination. All biopsies were compared with the baseline biopsies obtained before enrollment to determine whether regression or progression had occurred. Response was defined as regression of the lesion by two or more grades (CIN 2 to normal and CIN 3 to CIN 1 or normal). Subjects whose cervical biopsy demonstrated persistent CIN 3 at their 6-month visit or progression of their disease at any time during the clinical trial were viewed as treatment failures, removed from the study, and offered standard therapies. Other criteria for removal from the study included failure to complete scheduled tests or appointments, noncompliance with pill consumption, unacceptable toxicity, refusal or inability to continue the study, pregnancy, or the development of an invasive malignancy.

HPV Assessment. Cervical scrapings for HPV typing were collected in viral transport media (Digene, Silver Springs, MD) at the initial evaluation at UCI and at 3-month intervals thereafter. We were unable to obtain the cervical biopsy blocks from outside facilities to perform HPV typing on the original diagnostic material. Cervical scrapings at the time of enrollment (1–3 months after the diagnostic CIN 2–3 biopsy) were used for HPV assessment by PCR, as previously described (15). Briefly, DNA extracted from a cervical scraping was amplified with β -globin primers to confirm the presence of amplifiable DNA. Consensus primers in the *HPV L1* gene, *MY09/MY11*, (16) and type-specific primers for HPV 6, 16, and 18 as well as the reaction conditions, have been published previously (15). Every reaction set up contained appropriate positive and negative controls. The amplified PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide for detection of a visual product, and then Southern blot transferred to nylon membranes (MagnaNT; MSI) for hybridization overnight with 32 P-labeled random-primed probes for HPV 6, 16, and 18. The membranes were washed four times under stringent conditions with $2\times$ SSC with 0.1% SDS at 60°C and exposed to X-ray film with an intensifying screen at -80°C for 4 days. Samples positive with the consensus primers but negative with the HPV type-specific primers 6, 16, and 18 were analyzed by RFLP analysis of the consensus product as described (17, 18). In selected cases, if necessary, the PCR products were sequenced for final identification. Samples with multiple HPV types were also identified by RFLP analysis of the consensus product. Samples in which no HPV DNA was detected with either of the

primary sets were also amplified with additional consensus primers in the *E1* gene, *IU/WD0* (19), and products were subsequently sequenced for identification.

Degree of risk for progression of disease was grouped as follows: high (HPV 16, 18, and 45), intermediate (HPV 31, 33, 35, 39, 52, 56, and 58), and low (HPV 6, 11, 42, 43, 44, and 53). No risk category was assigned to patients in whom HPV was found to be present but either could not be identified or in which MM9 had been detected. Patients with more than one HPV type identified were assigned to the highest risk group of the viruses identified.

Micronutrient Measurement. Micronutrient levels of retinol, vitamin E, and β -carotene were measured from the serum at the initial visit, as well as at the 6-, 12-, 18-, and 24-month examinations, as described previously (20). Briefly, 250 μ l of 1% SDS in ethanol containing 0.1% butylated hydroxytoluene were added to a 0.25-ml aliquot of plasma. The samples were vortex-mixed for 60 s. Then, 500 μ l of hexane containing 0.1% butylated hydroxytoluene were added, vortex-mixed for 60 s, and centrifuged at $13,000 \times g$ for 1 min. The upper hexane layer was carefully removed and transferred to a microcentrifuge tube. The extraction was repeated once. The two-hexane layers were combined and dried under nitrogen. The extracts were analyzed using a HPLC system consisted of a Waters 600E multisolvent delivery system, a Waters 715 Ultra-WISP autoinjector, and a Waters 991 photodiode array detector. The vaginal micronutrients were measured in a similar fashion after vaginal irrigation with saline.

We implemented a QC/QA program to monitor the accuracy and consistency of our data routinely. Briefly, a QC sample was extracted along with every six samples and was analyzed by HPLC daily at the beginning and at the end of the six samples. The coefficient of variation of our between-day assays for the QC sample was $<8\%$ for all micronutrients analyzed. In addition, we participated in the QC/QA program for micronutrient analysis sponsored by the National Institutes of Standards and Technology from the time of its inception in 1984 until 1999. Our lab performed extremely well on all of the QC/QA round-robin studies carried out by the National Institutes of Standards and Technology.

Statistics. When the grant was originally written, it was anticipated that between 10 and 28% of subjects receiving placebo would show regression of their lesions compared with 30–50% of subjects receiving β -carotene. A sample size of 60/treatment arm was planned, because it would have at least 80% power to detect a 22% difference (from 28–50%) in response rates using a one-sided χ^2 test. An interim analysis was also planned to compare the arms after 60 patients were enrolled, because the study would have high power to detect extreme differences in response rates (10% versus 50%), which was considered a plausible outcome. A drop-out rate of 17% was anticipated. Early in the study, two refinements were made to the analysis plan; the interim analysis would be conducted at $\alpha = .005$ and the final analysis at $\alpha = .045$, and the tests would be two-sided. These choices lowered the final analysis power to 68% for a 22% difference in treatment response. No statistically significant difference between treatment arms was seen at the 6-month interim analysis, so we proceeded with the 24-month study. Because of changes in health care delivery in Southern California, which unfortunately limited patient access to clinical trials, patient accrual stagnated, and the decision was made to close the trial after 103 patients were randomized, further lowering the power to 61%.

Intention-to-treat principles were applied to the 25 patients

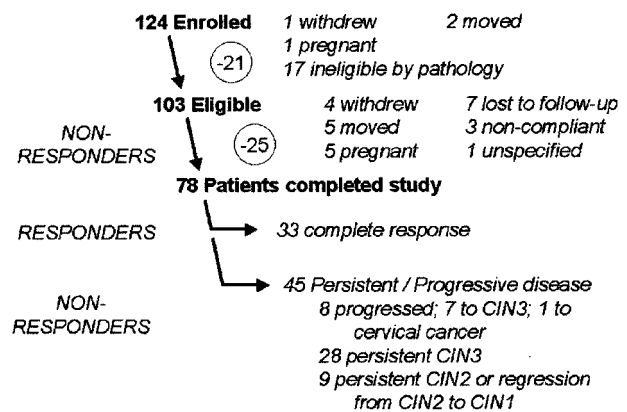


Fig. 1. Study subjects who were enrolled, randomized, and completed study.

who either withdrew or were withdrawn from the study; accordingly, all of these patients were recorded as nonresponders. The regression rates of CIN by treatment arm were analyzed using a Mantel-Haenszel χ^2 test stratified by CIN status at baseline. A Breslow-Day test was used to test for homogeneity of the odds ratio in the two strata. Fisher's exact test was used to analyze HPV subtypes by treatment, CIN, and response. Wilcoxon's rank-sum tests were used to compare micronutrient levels between groups. The data were analyzed using the SAS statistical package. All *P*s reported are two-tailed.

Results

Patient Characteristics. One hundred and twenty-four women were enrolled in the study between 1992 and 1996, 21 of whom were excluded because they either became pregnant (1), moved (2), or withdrew (1), or because reinterpretation of their initial cervical biopsies (17) did not confirm CIN 2 or CIN 3 (inflammation, condyloma, or CIN 1). The remaining 103 patients were included in the analysis. Twenty-five of 103 patients did not complete the study for a number of reasons [withdrew (4), noncompliant (3), lost to follow-up (7), pregnant (5), moved (5), and unspecified (1)] and were considered nonresponders for the analysis. (Fig. 1) Ten of these 25 patients were in the β -carotene treatment arm and 15 were in the placebo arm of the study. There were no significant differences between treatment groups in terms of marital status, age, race, HPV risk category (high-, intermediate-, or low-risk), OCP use, number of current smokers, or grade of lesion (Table 1). The mean age of the 103 study subjects was 29.8 years (range, 18.0–54.6 years). Racial groups included 49% Hispanic and 51% Caucasian. There was no statistically significant difference in the number of sexual partners ($P = 0.29$; β -carotene: mean, 6, median=3.5; placebo, mean, 5, median, 3) or number of pregnancies ($P = 0.87$; β -carotene: mean, 3, median, 2; placebo: mean, 3, median, 3) between the treatment arms.

Patient compliance was evaluated by the number of pills consumed as well as the measured serum β -carotene levels, which might have provided a more accurate reflection of β -carotene consumption. Patient compliance was 100% during the run-in period because all 124 enrolled patients consumed $>75\%$ of their pills. Mean compliance after randomization, as measured by the number of pills consumed by the 3- and 6-month examinations, was 97.7% and 95.4%, respectively. In addition, compliance, as determined by examining the distribution of serum β -carotene levels at enrollment and over the

Table 1 Characteristics of 103 patients eligible for analysis

Characteristics	Treatment group				P
	β -carotene		Placebo		
	n	%	n	%	
	51		52		
Grade					
CIN ^a 2 (baseline)	24	47	20	38	0.38
CIN 3 (baseline)	27	53	32	62	
Race					
Hispanic	23	45	27	52	0.56
White	28	55	25	48	
Age					
Age <30	31	61	28	54	0.55
Age \geq 30	20	39	24	46	
Marital status					
Single	27	53	22	42	0.33
Ever-married	24	47	30	58	
HPV risk category (n = 99)					
High	22	44	27	55	0.59
I/L/Unknown	15	30	12	25	
HPV (-)	13	26	10	20	
OCP use (n = 89)					
Yes	40	87	33	77	0.21
No	6	13	10	23	
Current Smokers (n = 88)					
Yes	15	33	14	33	0.94
No	30	67	29	67	
Number of sexual partners					
Mean	6		5		0.29
Median	3.5		3		
Number of pregnancies					
Mean	3		3		0.87
Median	2		3		

^a I, intermediate; L, low.

Table 2 Distribution of β -carotene levels based on treatment

Arm	Time (mo)	β -carotene serum values (ng/ml)					
		A ^a /n	0-99	100-499	500-999	1000-1999	2000+
P and BC	Baseline	91/91	69	18	1	1	2
P	6-24	109/44	77	18	8	2	4
BC	6-24	97/46	15	6	8	25	43

^a A, number of serum values that can be evaluated; P, placebo; BC, β -carotene.

24-month study period, is shown in Table 2. Samples for micronutrient levels at baseline were obtained from 91 of 103 enrollees. Ninety-six percent ($n = 87$ of 91) of the baseline values were <500 ng/ml. By contrast, a large majority ($n = 76$ of 97) of serum values from subjects on β -carotene supplementation had serum values that were equal to or exceeded 500 ng/ml. Thus, patients were judged to be "compliant" with their treatment if those on placebo had β -carotene levels <500 ng/ml, whereas those on supplementation had values ≥ 500 ng/ml. With this definition, 87 of 91 (96%) evaluable patients were compliant at baseline. The four other, "noncompliant," patients at baseline had serum β -carotene levels that were 830, 1734, 2562, and 6909 ng/ml, respectively, which most likely reflected other unreported supplementation of β -carotene intake. After treatment assignment, 95 of 109 (87%) of the placebo group serum assessments and 76 of 97 (78%) of the β -carotene group serum assessments were compliant. Nine of the 21 noncompliant values in the β -carotene group came from 3 individuals who consistently had low values.

Table 3 Response rates by treatment group and grade

Treatment group	% response (n)	
	No	Yes
β -carotene		
Overall	75% (38)	25% (13)
CIN 2	33% (17)	14% (7)
CIN 3	42% (21)	11% (6)
Placebo		
Overall	62% (32)	38% (20)
CIN 2	20% (10)	19% (10)
CIN 3	42% (22)	19% (10)

Response Evaluation. Seventy-eight patients completed the study, 33 of whom experienced lesion regression by two grades; 9 of whom had persistent CIN 2 or regression from CIN 2 to CIN 1; 8 of whom had progression to a higher grade, including 1 with a documented cervical cancer; and 28 of whom experienced persistent CIN 3 (Fig. 1). Interestingly, seven of eight patients whose lesions progressed were in the β -carotene treatment arm. Of the 28 who had persistent CIN 3, 14 were in the β -carotene treatment arm. The overall biopsy-confirmed response rate was 47% at 6 months, including 41% in the β -carotene arm and 52% in the placebo arm ($P = 0.33$). The overall response rate at 2 years was 32% (25% for β -carotene and 38% for placebo; $P = .21$). No difference in response was demonstrated between treatment groups after stratifying by CIN grade ($P = .13$; Table 3) If the 25 subjects who withdrew were considered as nonevaluable rather than nonresponders, the response rate at 24 months would have remained the same as it was at 6 months (47%). The initial grade of the lesion (CIN 2 versus CIN 3) was not associated with significantly different regression rates (39% versus 27%, respectively; $P = 0.29$) after 24 months.

Satisfactory specimens for HPV typing by PCR were obtained from cervical scrapings from 99 of 103 patients at enrollment. Seventy-seven percent ($n = 76$) of the cervical lesions were HPV-positive including 64% ($n = 49$) high-risk subtypes (HPV types 16, 18, and 45), 25% ($n = 19$) intermediate risk (HPV types 31, 33, 35, 39, and 52), 3% ($n = 2$) low-risk (HPV type 53), and 8% ($n = 6$) of an indeterminate risk with an unknown or MM9 type. HPV risk categories were not significantly different between both treatment arms at the time of enrollment ($P = 0.54$; Table 1). HPV risk category was significantly associated with response at 24 months; highest for women with no HPV detected (61%; 14 of 23), lower for intermediate-/low-risk (30%; 8 of 27), and lowest for high-risk (18%; 9 of 49) HPV groups ($P = .001$; Fig. 2).

Toxicity. Toxicity was graded from 0 to 4 based on the Southwest Oncology Group criteria. Minimal toxicity was experienced during the trial, with skin-yellowing as the most frequently reported side effect. Thirty-one patients reported skin-yellowing: 26 in the β -carotene arm and 5 in the placebo arm. No patients experienced greater than grade 2 skin-yellowing. There were no reports of vomiting or anorexia in any patient. One patient in the placebo arm complained of 3-4 loose stools/day (grade 1) between her 3-month and 6-month visits. Two patients in the placebo group complained of fatigue that did not interfere with their activities of daily living (grade 1). One patient on β -carotene supplementation experienced grade 1 nausea at 15 and 21 months.

Effect of Micronutrients. Baseline serum levels of vitamin A, β -carotene, and vitamin E were available for 91 patients and

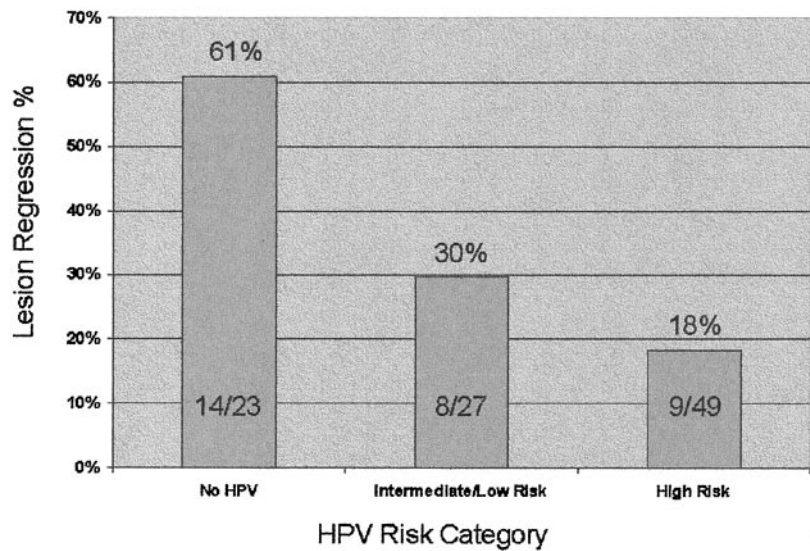


Fig. 2. HPV Risk Category and Lesion Regression (24 months).

Table 4 Serum micronutrient levels at baseline and in follow-up in both treatment groups

Micronutrient	Time (mo)	Arm	n	Interquartile			P ^a
				Median	Range	Range	
BC ^b	0	BC	46	168	81–256	32–6909	.33
	P	45	128	91–217	19–376		
BC	6–24	BC	97	1946	758–3224	34–6278	.0001
		P	109	156	91–305	6–3662	
Ret	0	BC	46	461	373–576	239–1388	.78
	P	45	486	375–611	253–938		
Ret	6–24	BC	97	453	375–595	58–931	.59
		P	109	463	370–554	89–1033	
Vit E	0	BC	46	10348	8176–12040	1930–19335	.88
	P	45	10554	8471–11869	4239–20805		
Vit E	6–24	BC	96	10260	8294–12694	4123–30802	.39
		P	108	10392	8608–13352	4179–38040	

^a P_s based on the Wilcoxon's rank-sum test with normal scores.

^b BC, β -carotene, Ret, retinol, Vit E, Vitamin E, P, placebo.

were similar between treatment arms. Table 4 shows the micronutrient levels at baseline (time 0) and over the study period (time 6–24 months). The median baseline β -carotene serum levels for the β -carotene and placebo treatment groups were 168 ($n = 45$) ng/ml and 128 ($n = 46$), respectively. The median baseline retinol levels for the β -carotene and the placebo treatment groups were 461 and 486 ng/ml, respectively, and the median baseline vitamin E levels for the β -carotene and placebo treatment groups were 10348 and 10554 ng/ml, respectively. On the other hand, there was a statistically significant difference in the median β -carotene serum levels in the β -carotene treatment arm (1946 ng/ml) versus the placebo arm (154 ng/ml) when measured from samples obtained between months 6 and 24 of the study ($P = 0.0001$). The median serum levels of retinol between 6 and 24 months in the β -carotene and placebo treatment groups were 453 ng/ml and 463 ng/ml, respectively, which were essentially the same as the baseline levels. Likewise, median vitamin E levels measured from samples obtained between 6 and 24 months in the 91 patients were 10260 ng/ml and 10392 ng/ml in the treatment and placebo groups, respectively, which did not vary significantly from pretreatment levels.

Using logistic regression on patients who had not dropped out before 6 months, the probability of CIN regression at 6 months as well as negatively correlated with baseline retinol levels ($P = .03$; $n = 82$). Baseline serum vitamin E or β -carotene levels did not influence the 6-month regression rate. No statistically significant correlations were identified between baseline or 6-month serum retinol levels and response at 24 months.

A statistically significant increase in the mean vaginal concentrations of β -carotene in women consuming β -carotene ($P = 0.0001$) was observed, and this positively correlated with serum β -carotene levels ($P = 0.011$). No significant difference in vaginal β -carotene levels was seen in women whose cervical lesions regressed versus those whose lesions persisted or progressed.⁴

⁴ McHale, M. T., Monk, B. J., Keefe, K. A., Schell, M. J., Peng, Y. M., and Berman, M. L. Influence of baseline and twenty-four-month serum and tissue micronutrient levels on the spontaneous regression of CIN II and III.

Discussion

Primary prevention of cervical disease requires women to avoid high-risk sexual activity, which is associated with high rates of preinvasive and invasive cervical cancer. Secondary prevention of cervical cancer is accomplished through periodic Pap smear screening to detect and permit treatment of preinvasive cervical lesions. This study investigates another form of secondary prevention of cervical cancer, chemoprevention, through the possible role of β -carotene in causing regression of biopsy-proven, cervical squamous intraepithelial lesions. Briefly, three main conclusions can be drawn from this study: (a) β -carotene supplementation does not result in regression of high-grade biopsy-proven cervical lesions; (b) women who had a biopsy-proven CIN 2–3, but who had no HPV detected in their cervical scrapings at enrollment (1–3 months after biopsy), were more likely to undergo regression; and (c) a higher rate of regression at 6 months was associated with women whose pretreatment retinol levels were lower.

This 24-month analysis did not demonstrate enhanced regression of CIN 2 or 3 lesions in women who consumed β -carotene over those taking a placebo. In fact, there was a higher (though nonsignificant) regression rate in the placebo group (38%) than in the β -carotene group (25%) in this trial. The negative results may stem from our low projected power of 61% and the unexpectedly high dropout rate of 24%, with only 78 of 103 women randomized completing the study. It is likely, however, that the regression rates observed represent spontaneous regression of high-grade cervical lesions perhaps affected by several diagnostic biopsies; consequently, a larger sample size might not have made a difference. Because patients were blinded to their treatment assignment, it is unlikely that changes in behavior such as diet or increased supplementation would have accounted for the higher rates of regression observed in women randomized to the placebo arm. In addition, it is also unlikely that the placebo itself resulted in increased regression of the high-grade CIN.

The reported association between CIN regression and β -carotene supplementation in this trial and in other series suggests that oral supplementation with β -carotene probably does not lead to regression of CIN lesions beyond that which results from spontaneous regression. Fairley *et al.* (10) found no difference in regression of CIN 1 lesions after 12 months of follow-up in women who received 30 mg of β -carotene daily *versus* placebo. Similarly, de Vet *et al.* (13) found no regression of CIN lesions with 10 mg daily supplementation of β -carotene *versus* placebo. Romney *et al.* (11) actually reported significantly lower regression rates of CIN 1–3 lesions in women who were randomized to receive 30 mg of daily β -carotene *versus* the placebo group after 9 months of follow-up (23.1% *versus* 46.7%, respectively; $P = .039$). Likewise, Mackerras *et al.* (12) recently reported slightly increased progression of squamous atypia and CIN 1 in women randomized to 30 mg of daily β -carotene *versus* no β -carotene. These observations are consistent with the results of the current 24-month randomized clinical trial. Only one placebo-controlled clinical trial using a related compound, β -transretinoic acid applied topically to the cervical tissue, demonstrated a positive effect on CIN 2 regression but not CIN 3 regression after 15 months (14).

The current study found a higher rate of regression in women with no detectable HPV compared with HPV-positive subjects who were assessed for HPV by PCR 1–3 months after the diagnostic CIN 2–3 biopsy. It is unlikely that the high rate of (–) women enrolled is attributable to technical factors, as the MY09/MY11 PCR primers have been demonstrated to be ex-

tremely sensitive for detection of most HPV types associated with cervical lesions (15), and all viral negative samples were also amplified with second set of consensus primers directed against a different HPV gene (*E1*). We were unsuccessful in obtaining the blocks of the original biopsies of the HPV-negative women from outside facilities to test. Supporting the spontaneous resolution of the CIN lesions, 50% of the women with no HPV detected at enrollment had a concurrent Pap smear that was read as normal, reactive, or atypical squamous cells of undetermined significance (ASCUS). The high rate of HPV-negativity in this population of women with documented CIN 2–3 lesions may be related to stimulation of their immune system by the previous biopsy with viral clearance. Because all women had colposcopic evidence of persistent disease at enrollment, the morphological changes may take longer to resolve. Therefore, a persistent, abnormal Pap smear should be required in future chemoprevention trials for enrollment, in addition to a positive colposcopy, to attempt to control for cervical lesion regression that may actually be caused by the biopsy. In this study, in the HPV-positive women, regression of preneoplastic lesions was seen least frequently in women with high-risk HPV subtypes and by a higher regression rate in women with intermediate- and low-risk HPV subtypes, which is consistent with previous studies (21, 22).

Finally, complex interactions between various micronutrients exist, are not well understood, and might explain the failure of β -carotene to increase the CIN regression rate over that of placebo. As anticipated, vitamin E and retinol levels were similar between treatment arms at baseline and throughout the study. Furthermore, β -carotene serum levels were similar between treatment arms at baseline but, as expected, were significantly higher in women supplemented with β -carotene throughout the study. In addition we observed an expected, positive correlation between vaginal and serum β -carotene levels with supplementation. Despite this positive correlation, however, we did not observe an increase in cervical lesion regression in women randomized to daily β -carotene supplementation. We did observe a correlation between baseline retinol levels and lesion regression at 6 months, with low initial levels associated with a greater probability of lesion regression. Because β -carotene is normally converted to retinol after consumption, it is possible that increased bioavailability of retinol (which is not measurable) or a change in serum retinol levels in women who are more deficient in this micronutrient, may somehow lead to regression of their cervical lesions. Supporting this hypothesis is the observation that cancer-related mortality is decreased when study participants in China found to be deficient in various vitamins and minerals are supplemented with vitamins A and E and selenium (23). Our finding that regression of cervical lesions was most likely in women whose pretreatment plasma retinol levels were low suggests that a subgroup of women with low vitamin A and β -carotene intake should be targeted for future chemoprevention trials for cervical cancer. An analysis examining the interrelationships among vitamin E, retinol, and β -carotene has been completed and will be reported in a separate manuscript (1).

In conclusion, this 24-month analysis failed to demonstrate benefit or harm to women with CIN 2 or 3 treated for 24 months with placebo or β -carotene. Side effects were similar and equal in both arms. The regression rate of lesions in patients randomized to receive placebo was 38% *versus* 25% in the β -carotene arm. If one assumes that regression in both arms represents “spontaneous regression,” the overall spontaneous regression rate after 24 months in this trial was 32%. If patients who dropped out of the trial were considered as nonevaluable

rather than as nonresponders, the overall regression rate was 47%. The observed regression rate was highest in HPV-negative subjects. The regression rate at 6 months was higher in women with low pretreatment retinol levels. However, given the dropouts in this study, the relationship weakened at 24 months. These results may allow us to target subgroups of women for future chemoprevention trials. Cervical biopsies and the associated cell-mediated immune response may be partly responsible for the high rate of regression of high-grade lesions and the low rate of detection of HPV in the initial cervical scrapings. Future chemoprevention studies should require a persistent abnormal Pap smear for enrollment so that we can assess the true rate of spontaneous regression as well as the true effect of the chemoprevention agent being evaluated.

Acknowledgments

We acknowledge Sandhya Upasani for her statistical support as well as Phyllis Villaverde for interviewing study subjects.

References

- Palan, P. R., Mikhail, M. S., Goldberg, G. L., Basu, J., Runowicz, C. D., and Romney, S. Plasma levels of β -carotene, lycopene, canthaxanthin, retinol, and γ -tocopherol in cervical intraepithelial neoplasia and cancer. *Clin. Cancer Res.*, 2: 181–185, 1996.
- McPherson, R. S. Nutritional factors and the risk for cervical dysplasia. *Am. J. Epidemiol.*, 130: 830–831, 1989.
- Block, G., Patterson, B., and Subar, A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer*, 18: 1–29, 1992.
- Block, G. The data support a role for antioxidants in reducing cancer risk. *Nutr. Rev.*, 50: 207–213, 1992.
- Romney, S. L., Palan, P. R., Dutttagupta, C., Wassertheil-Smoller, S., Wylie, J., Miller, G., Slagle, N. S., and Lucido, D. Retinoids and the prevention of cervical dysplasias. *Am. J. Obstet. Gynecol.*, 141: 890–894, 1981.
- Wassertheil-Smoller, S., Romney, S. L., Wylie-Rosett, J., Slagle, N. S., Miller, G., Lucido, D., Dutttagupta, C., and Palan, P. R. Dietary vitamin C and uterine cervical dysplasia. *Am. J. Epidemiol.*, 114: 714–724, 1981.
- Palan, P. R., Romney, S. L., Mikhail, M., Basu, J., and Vermund, S. H. “Decreased plasma β -carotene levels in women with uterine cervical dysplasias and cancer” (Letter). *J. Natl. Cancer Inst.*, 80: 454–455, 1988.
- Batieha, A. M., Armenian, H. K., Norkus, E. P., Morris, J. S., Spate, V. E., and Comstock, G. W. Serum micronutrients and the subsequent risk of cervical cancer in a population-based nested case-control study. *Cancer Epidemiol. Biomark. Prev.*, 2: 335–339, 1993.
- Muto, Y., Fujii, J., Shidoji, Y., Moriwaki, H., Kawaguchi, T., and Noda, T. Growth retardation in human cervical dysplasia-derived cell lines by β -carotene through down-regulation of epidermal growth factor receptor. *Am. J. Clin. Nutr.*, 62: 1535S–1540S, 1995.
- Fairley, C. K., Tabrizi, S. N., Chen, S., *et al.* A randomized clinical trial of β -carotene vs. placebo for the treatment of cervical HPV infection. *Int. J. Gynecological Cancer*, 6: 225–230, 1996.
- Romney, S. L., Ho, G. Y., Palan, P. R., Basu, J., Kadish, A. S., Klein, S., Mikhail, M., Hagan, R. J., Chang, C. J., and Burk, R. D. Effects of β -carotene and other factors on outcome of cervical dysplasia and human papillomavirus infection. *Gynecol. Oncol.*, 65: 483–492, 1997.
- Mackerras, D., Irwig, L., Simpson, J. M., Weisberg, E., Cardona, M., Webster, F., Walton, L., and Ghersi, D. Randomized double-blind trial of β -carotene and vitamin C in women with minor cervical abnormalities. *Br. J. Cancer*, 79: 1448–1453, 1999.
- de Vet, H. C. W., Knipschild, P. G., Willebrand, D., Schouten, H. J. A., and Sturmans, F. The effect of β -carotene on the regression and progression of cervical dysplasia: a clinical experiment. *J. Clin. Epidemiol.*, 44: 273–283, 1991.
- Meyskens, F. L., Jr., Surwit, E., Moon, T. E., Childers, J. M., Davis, J. R., Dorr, R. T., Johnson, C. S., and Alberts, D. S. Enhancement of regression of cervical intraepithelial neoplasia II (moderate dysplasia) with topically applied all-*trans*-retinoic acid: a randomized trial. *J. Natl. Cancer Inst.*, 86: 539–543, 1994.
- Monk, B. J., Cook, N., Ahn, C., Vasilev, S. A., Berman, M. L., and Wilczynski, S. P. Comparison of the polymerase chain reaction and Southern blot analysis in detecting and typing human papilloma virus deoxyribonucleic acid in tumors of the lower female genital tract. *Diagn. Mol. Pathol.*, 3: 283–291, 1994.
- Ting, Y., and Manos, M. M. Detection and typing of genital human papillomaviruses. In: M. A. Innis, D. G. Gelfand, J. J. Sninsky, and T. J. White (eds.), *PCR Protocols: A Guide to Methods and Applications*, pp. 356–367. San Diego, CA: Academic Press, Inc., 1990.
- Wilczynski, S. P., Oft, M., Cook, N., Liao, S. Y., and Iftner, T. Human papillomavirus type 6 in squamous cell carcinoma of the bladder and cervix. *Hum. Pathol.*, 24: 96–102, 1993.
- Meyer, T., Arndt, R., Stockfleth, E., Flammann, H. T., Wolf, H., and Reischl, U. Strategy for typing human papillomaviruses by RFLP analysis of PCR products and subsequent hybridization with a generic probe. *Biotechniques*, 19: 632–639, 1995.
- Gregoire, L., Arella, M., Campione-Piccardo, J., and Lancaster, W. D. Amplification of human papillomavirus DNA sequences by using conserved primers. *J. Clin. Microbiol.*, 27: 2660–2665, 1989.
- Peng, Y. S., Peng, Y. M., McGee, D. L., and Alberts, D. S. Carotenoids, tocopherols and retinoids in human buccal mucosal cells: intra- and interindividual variability and storage stability. *Am. J. Clin. Nutr.*, 59: 636–643, 1994.
- Stoler, M. H. A brief synopsis of the role of human papillomaviruses in cervical carcinogenesis. *Am. J. Obstet. Gynecol.*, 175: 1091–1098, 1996.
- Schiffman, M. H., and Brinton, L. A. The epidemiology of cervical carcinogenesis. *Cancer (Phila.)*, 76: 1888–1901, 1995.
- Yu, Y., Taylor, P. R., Li, J. Y., Dawsey, S. M., Wang, G. Q., Guo, W. D., Wang, W., Liu, B. Q., Blot, W. J., and Shen, Q. Retrospective cohort study of risk factors for esophageal cancer in Linxian, People’s Republic of China. *Cancer Causes Control*, 4: 195–202, 1993.