

# A Randomized Double-Blind Placebo-Controlled Trial of Fruit and Vegetable Concentrates on Intermediate Biomarkers in Head and Neck Cancer

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## Abstract

**Background.** Head and neck cancer (HNC) patients are at an increased risk for developing second primary tumors (SPTs). Diets rich in fruits and vegetables (FVs) may lower HNC risk. FV concentrates may offer a potential alternative to increasing FV intake. **Methods.** We conducted a randomized, double-blind, placebo-controlled trial to evaluate whether Juice PLUS+ (JP; a commercial product with multiple FV concentrates) has an effect on p27 and Ki-67, biomarkers associated with the risk of SPTs. During 2004–2008, we randomized 134 HNC patients to 12 weeks of JP (n = 72) or placebo (n = 62). Oral cavity mucosal biopsies and whole blood were obtained at baseline and after 12 weeks. All participants were given the opportunity to receive JP for 5 years following the end of the intervention period, and they were followed yearly for the development of SPTs. **Results.** After 12 weeks, patients on JP had significantly higher serum  $\alpha$ -carotene ( $P = .009$ ),  $\beta$ -carotene ( $P < .0001$ ), and lutein ( $P = .003$ ) but did not differ significantly in p27 ( $P = .23$ ) or Ki-67 ( $P = .95$ ). JP use following the initial 12-week trial was not significantly associated with SPT prevention. **Conclusions.** Despite increased serum micronutrient levels, our results do not suggest a clinical benefit of JP in HNC patients. Future studies should focus on longer intervention periods and/or modified supplement formulations with demonstrated chemopreventive properties.

## Keywords

fruit/vegetable concentrate, surrogate end point biomarkers, head and neck cancer, p27, Ki-67

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## Introduction

Head and neck cancer (HNC) patients have a significantly higher rate of lipid peroxidation and DNA damage<sup>1–3</sup> and are at an increased risk of developing second primary tumors (SPTs). Continued smoking and excess alcohol consumption contribute to increased oxidative stress and cancer risk.<sup>4</sup> Low p27 (a cyclin-dependent kinase inhibitor; critical in cell cycle regulation) and/or high Ki-67 (cell proliferation associated nuclear protein) has been shown to correlate with tumor progression and poor prognosis in these patients.<sup>5–8</sup> To improve survival, an effective program of chemoprevention for second malignancies is essential in HNC patients.

Diets rich in fruits and vegetables (FVs) decrease HNC risk<sup>9,10</sup> and reduce markers of cellular oxidative damage.<sup>11</sup>

However,  $\geq 75\%$  of the US population consumes FVs below the recommended amounts for their age and gender group.<sup>12</sup> Smoking is one of the most compelling risk factors for the development of HNC,<sup>13</sup> and intake of FVs is even lower among smokers.<sup>14–16</sup> Lower FV intake<sup>14–16</sup> and free radicals

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from cigarette smoke<sup>17-20</sup> may contribute to decreased plasma antioxidant levels. Additionally, smoking appears to elicit dose-related higher Ki-67 labeling indices in the bronchial epithelia of active smokers.<sup>21</sup> Many clinical trials of vitamin and mineral supplements have failed to demonstrate a strong association between isolated nutrients and the risk of HNC,<sup>22-27</sup> indicating that perhaps a single nutrient cannot replicate the synergistic effect of the numerous chemoprotective compounds present in whole foods such as FVs. FV concentrates may offer a potential alternative for increasing vitamin, mineral, and phytochemical intakes.

Several small studies of FV concentrate supplementation in healthy participants have shown reduction of DNA damage, lower lipid peroxide levels, and a boost in the immune response.<sup>28-30</sup> Juice PLUS+ (JP), a FV concentrate significantly increased serum antioxidants (such as  $\beta$ -carotene, lutein, lycopene, retinol, vitamin C,  $\alpha$ -tocopherol) in healthy participants.<sup>28,31,32</sup> However, to our knowledge, the efficacy of FV concentrates has not been evaluated in HNC patients. We undertook this study to evaluate the effects of JP (NSA International, Memphis, TN) on surrogate end point biomarkers (SEBs) associated with the development of SPTs in patients with previous HNC. The primary and secondary end points were p27 expression and Ki-67 proliferation rates, respectively. We also evaluated whether the augmentation of SEBs by JP is influenced by other factors, such as age, tumor site, and stage.

## Methods

### Study Population

This randomized, double-blind, placebo-controlled trial to assess the effects of JP on SEB in patients with previous HNC was conducted between January 2004 and October 2008. Patients were followed for clinical events through January 2014. Patients >18 years old and free of HNC (minimum 6 months and maximum 3 years) following completion of treatment for squamous cell carcinoma of the upper aerodigestive tract, with life expectancy of >6 months with no synchronous tumors, a negative serum pregnancy test within 10 days of registration, normal hematological parameters (hemoglobin  $\geq 10$  g/dL; white blood cells  $\geq 3000/\mu\text{L}$ ; platelets  $\geq 100000/\mu\text{L}$ ), adequate liver (bilirubin  $\leq 1.5$  mg/dL, serum glutamic oxaloacetic transaminase (SGOT)  $\leq 40$  U/L, serum glutamic pyruvic transaminase (SGPT)  $\leq 56$  U/L) and renal function (creatinine  $\leq 1.5$  mg/dL), Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 to 1, and no consumption of JP within 6 months prior to enrollment were eligible to participate.

Candidates were ineligible if they had a concomitant malignancy other than curatively treated HNC within the past 5 years (except non-melanoma skin cancer and in situ carcinoma of the cervix), a serious medical or psychiatric

illness that prevented informed consent, or persistent nausea (>grade 2 by National Cancer Institute [NCI] Common Toxicity Criteria [CTC] Version 3.0) or if they consumed mega doses of supplements such as >10000 IU or 3440  $\mu\text{g}$  vitamin A, >1000 mg vitamin C, or >800 IU or 537 mg vitamin E daily within the past 2 months.

Patients were randomized within strata (tumor stage I, II, III, or IV; by tobacco and alcohol use) to JP or placebo with equal probability. The institutional review board at Wake Forest Baptist Health reviewed and approved the study protocol (trial registration ID: NCT00064298; Comprehensive Cancer Center of Wake Forest University Protocol no. 60A02). The research protocol was reviewed and approved at the other participating Research Base sites (Southeast Cancer Control Consortium, Upstate Carolina CCOP, Central Illinois CCOP, East Carolina University, Harbin Clinic Radiation Oncology, Greater Phoenix CCOP, Alamance Cancer Center and Louisiana State University MBCCOP). Participants were recruited at multiple sites and recruitment sites were not required to record screening data. Informed consent was obtained from all study participants. Participants received a \$10 gift certificate after baseline visit and a \$40 gift certificate on study completion. Beyond provision of JP capsules, NSA International (Memphis, TN) had no role in the study design, data collection, analysis, and/or interpretation.

### Data Collection and Follow-up

Research nurses conducted screening interviews to obtain information on medical history, tobacco and alcohol use, and PS and to administer the baseline HNC questionnaire. After the interview, the research nurse collected blood for screening evaluation. Candidates with normal blood chemistry were invited to initiate the 1-week run-in period, which involved taking placebo pills as prescribed and maintaining a complete and accurate medication diary. The pill bottle was returned to the nurse who counted pills and compared with the diary. Study participants with >75% compliance were randomly assigned to receive either JP or placebo for 12 weeks. Medication diary and pill counts were collected at baseline and at the end of 12 weeks of intervention.

### Study Supplementation

JP is a proprietary blend of dried FV powders extracted from apples, oranges, pineapples, papaya, cranberries, and peaches (orchard blend); carrots, parsley, beets, broccoli, kale, cabbage, spinach, and tomatoes (garden blend); and citrus bioflavonoids, calcium ascorbate and carbonate, *Lactobacillus acidophilus*, d- $\alpha$  tocopherol, mixed tocopherols, *Dunaliella salina*,  $\beta$ -carotene, and folic acid. The nutritional profile of JP capsules is provided in Table 1. The FV concentrates are

**Table 1.** Nutrition Composition of Juice PLUS+.

Nutrient	Garden Blend <sup>a</sup> (Blended Vegetables)		Orchard Blend <sup>a</sup> (Blended Fruit)		Total Daily Intake <sup>b</sup>	
	Actual	Percentage Daily Value	Actual	Percentage Daily Value	Actual	Percentage Daily Value
Calories (kcal)	5	0	5	0	10	
Total fat (g)	0	0	0	0	0	
Cholesterol (mg)	0	0	0	0	0	
Sodium (mg)	10	<1	5		15	
Total carbohydrate (g)	1	<1	1		2	
Dietary fiber (g)	<1	2	<1	1	<2	3
Sugars (g)	<1		<1		<2	
Protein (g)	<1		<1		<2	
Vitamin A (100% as $\beta$ -carotene)		140		110		250
Vitamin E		80		70		150
Vitamin C		70		320		390
Folate		70		35		105
Calcium		4		2		6

<sup>a</sup>Nutrition information per 2 capsules each.

<sup>b</sup>1 Fruit and vegetable capsule each in the morning and evening.

encapsulated separately in hard gelatin capsules to provide 850 mg of fruit powder and 750 mg of vegetable powder per capsule. Test supplements were obtained from the same product lot. Based on the guidelines established by the Food and Drug Administration, FV concentrates are generally recognized as safe. JP has previously been studied in healthy participants<sup>29,31,33</sup> and women with ovarian cancer, with no reported toxic effects.<sup>34</sup> Consequently, for this study, we chose the JP dose previously evaluated (1 capsule each of orchard and garden blend administered twice daily).

Participants received 2 capsules of either JP or placebo in the morning and 2 in the afternoon/evening. If the participant was unable to swallow the pills, the capsules were opened and the contents sprinkled over food, and the participant was instructed to consume all the food containing the capsule contents. The capsule contents of JP and placebo were similar in appearance, so that the participants could not discern their group assignment even if the capsules were opened. Study participants were instructed to maintain their usual dietary habits and lifestyle during the trial. Telephone interviews were conducted at weeks 1, 2, 3, 4, and 8 of supplementation to evaluate side effects and tolerance.

Storage and distribution of JP and placebo pills was handled by Biologics, Inc (Raleigh, NC). On notification of participant enrollment in the study, Biologics, Inc shipped a 12-week supply of either JP or placebo to the participant's address.

### Sample Collection and Laboratory Procedures

At baseline, following a complete head and neck examination by an otolaryngologist or a radiation oncologist, buccal

mucosa biopsies were taken from the left side of the mouth. This area was chosen for its accessibility. At 12 weeks, a buccal mucosa biopsy was obtained from the right side of the mouth. Biopsies were obtained under local anesthesia. A single biopsy measuring a minimum 1 cm  $\times$  2 mm was obtained and divided into 4 pieces. One piece of the biopsy was immediately fixed in 10% buffered formalin. The other 3 biopsy sections were frozen in dry ice. Approximately 30 mL whole blood was obtained, tubes were centrifuged, and the separated serum was stored at  $-70^{\circ}\text{C}$  until analyzed.

### Serum Micronutrient Levels

The samples were processed under subdued yellow/orange lighting and the lipid-soluble vitamin assays were conducted based on methods adopted from Hess et al<sup>35</sup> and Aebischer et al.<sup>36</sup> For this, 200  $\mu\text{L}$  of plasma or standard solution and internal standard was deproteinized with 1 mL ethanol followed by 2 mL hexane stabilized with 30 mM butylated hydroxy toluene (BHT) and buffer. Samples were extracted twice with hexane/BHT under argon. The combined organic layers were dried under nitrogen and reconstituted with 200  $\mu\text{L}$  of 30% methanol, 30% dioxane, and 40% acetonitrile. Two detectors were required to monitor the necessary wavelengths to quantify the analytes at their absorbance maxima to ensure sensitivity. Analytes were separated on a Beckman ODS 5- $\mu\text{m}$ , 250  $\times$  4.6 mm<sup>2</sup> column (Beckman Coulter Inc, Brea, CA). The isocratic mobile phase consisted of 680 mL acetonitrile, 220 mL tetrahydrofuran, 68 mL methanol, and 28 mL of a 1% ammonium acetate solution. The analyte limits of detection were

determined by Hess et al<sup>35</sup>:  $\alpha$ -carotene 10  $\mu\text{g/L}$ ,  $\beta$ -carotene 10  $\mu\text{g/L}$ ,  $\beta$ -cryptoxanthin 10  $\mu\text{g/L}$ , lycopene 5  $\mu\text{g/L}$ , retinol 20  $\mu\text{g/L}$ ,  $\alpha$ -tocopherol 0.05 mg/L, and  $\gamma$ -tocopherol 0.05 mg/L. A 7-point standard curve along with low and high controls was run daily with participant samples. Assays where the controls varied by >10% of expected values were repeated.

### **Histopathological and Immunohistochemistry Evaluation**

Standard microscopic examination of all tissue biopsies was done using cytologic and architectural pathologic criteria to differentiate reactive from dysplastic changes. Formalin-fixed tissue was paraffin embedded, and 4- $\mu\text{m}$  sections were cut and put onto slides (Baxter, Charlotte, NC). These sections were air dried, deparaffinized with xylene, hydrated, and antigen retrieved, and then the slides were stained, using an automated stainer for expression of p27 and Ki-67 and a streptavidin-biotin peroxidase technique with an anti-human p27 mouse monoclonal antibody (Transduction Laboratories, Lexington, KY) or an anti-human Ki-67 mouse monoclonal antibody (Immunotech, France). The slides were counterstained with Gill's hematoxylin and analyzed for percentage of cells stained and stain intensity. In each study, a negative control was also run, where the primary treatment contained no primary antibody.

### **Toxicity and Response Evaluation Criteria**

Toxicity was determined using the NCI-CTC, v3.0 for toxicity and adverse event reporting.<sup>37</sup> The study chairman was notified immediately by telephone of any unexpected, life-threatening (grade 4), or fatal (grade 5) adverse event, with an attribution of possible, probable, or definite cause. Queried toxicities included anorexia, nausea, vomiting, diarrhea, fatigue, fever, and heartburn. No toxicity related to JP was expected, but because gastrointestinal toxicity was a potential concern, the research nurse conducted telephone interviews weekly during the first month of the intervention. Participants were asked to rate symptoms of gastrointestinal upset on a 5-point scale. Participants who experienced  $\geq$ grade 3 symptoms would have been withdrawn from the study. The institutional review board was to be notified of any study-related (JP) adverse events. Response to supplements was determined by comparing p27 and Ki-67 measured at baseline and after 12 weeks of supplementation.

### **Statistical Analysis**

The primary objective of this study was to assess the effects of JP on p27, the biomarker quantitating cell-cycle regulation. The sample size was based on a *t*-test comparison between treatment groups at the 5%, 2-sided level of

significance. The sample sizes per group needed to detect a 20% relative difference between groups (assuming a mean  $\pm$  SD p27 value in the control group of  $34.68 \pm 11.41$  from the Mineta et al<sup>7</sup> study) with 80%, 85%, and 90% power were 46, 52, and 60, respectively. Assuming a 40% drop-out rate, we planned to accrue 200 participants to provide 90% power. The drop-out rate was much lower than anticipated (only 8%), so accrual was terminated after 134 participants. Patients were stratified by tobacco and/or alcohol use and primary tumor stage (stage I, II, III, and IV) and randomized within strata to JP or placebo groups with equal probability using variably sized permuted blocks. Block sizes of varying length were determined randomly to ensure that future assignments could not be inferred from past assignments. The randomization allocation sequence generated by a statistician at the Wake Forest University Comprehensive Cancer Center was stored as a database table and accessed via an on-line registration/randomization facility. Because participants were enrolled at multiple sites across the United States, research staff at these sites enrolled participants, and group assignment was automatically done by the randomization facility on registration. Participants, care givers, and those who did the outcome assessments were all blinded to the intervention.

Participant characteristics and outcome measures are summarized using descriptive statistics for each treatment group. Because of some bad sections and missing data, some participants had outcome data at one time point but not the other. A mixed-effects, repeated-measures model fit using an unstructured covariance matrix was used to assess the effect of treatment arm on the primary (p27) and secondary outcome measures (Ki-67), using all data collected. This model was constrained, so that the baseline estimate was the same in both groups, as appropriate for randomized trials.<sup>38</sup> Posttreatment least-square (LS) means and standard errors for each treatment arm were estimated along with 95% CIs for the treatment differences. Analysis of covariance (ANCOVA) was then used to assess the effect of JP after adjusting for baseline characteristics (age, sex, race, body mass index [BMI], performance status (PS), stage, and tobacco and alcohol use), the effect of characteristics on the change in p27 and Ki-67, and whether the effect of JP was influenced by these characteristics.

All participants were given the opportunity to receive JP for 5 years following the end of the intervention. Participants were followed yearly for those 5 years for the development of SPTs and to record JP use. In an unplanned, exploratory analysis, we assessed the association between JP use and the risk of SPTs. We used self-reported JP intake to create event history records and the occurrence of events. For example, a participant may have used JP for the first 2 years and not for the last 3 years of follow-up. A Cox proportional hazards regression model utilizing a counting process input<sup>39</sup> (which utilizes multiple start/stop records per

**Table 2.** Summary of Participant Characteristics in the Juice PLUS+ Supplementation in the Head and Neck Cancer Trial.

	Juice PLUS+	Placebo
	n (%)	n (%)
Participants	72 (100)	62 (100)
Age		
Median (range)	58 (30-82)	59 (41-82)
≥60 years	31 (43)	31 (50)
Gender		
Female	11 (15)	10 (16)
Male	61 (85)	52 (84)
Race/Ethnicity		
Black	4 (6)	7 (11)
Hispanic	7 (10)	2 (3)
White	58 (81)	53 (85)
Other/Unknown	3 (4)	0 (0)
Body mass index		
Median (range)	25.0 (16.5-39.8)	25.1 (16.2-36.6)
Underweight/Normal (<25 kg/m <sup>2</sup> )	35 (49)	28 (45)
Overweight (25-29.9 kg/m <sup>2</sup> )	30 (42)	25 (40)
Obese (≥30 kg/m <sup>2</sup> )	7 (10)	9 (15)
Performance status		
0	60 (83)	53 (85)
I	12 (17)	9 (15)
Stage <sup>a</sup>		
I-II	26 (36)	20 (32)
III-IV	46 (64)	42 (68)
Treatment		
Surgery alone	11 (15)	10 (16)
Radiation therapy alone	4 (6)	3 (5)
Surgery + Radiation therapy	12 (17)	18 (29)
Radiation + Chemotherapy	22 (31)	15 (24)
Surgery + Radiation + Chemotherapy	23 (32)	16 (26)
Lifestyle characteristics		
Alcohol use <sup>a</sup>	32 (44)	27 (44)
Tobacco use <sup>a</sup>	16 (22)	14 (23)

<sup>a</sup>Factors used for stratification.

patient) was used to assess the effect of JP on the risk of secondary events.

## Results

Previously diagnosed HNC patients (n = 134) were accrued and randomly assigned to receive either JP (n = 72) or a placebo (n = 62). Participant ages ranged from 30 to 82 years, with a median age of 59 years. Most participants were male (84%) and Caucasian (83%), and 12% (n = 16) were obese

(BMI ≥ 30 kg/m<sup>2</sup>). In all, 22% (n = 30) still used tobacco products, and 44% (n = 59) continued to use alcohol (Table 2). Participant characteristics were similar between treatment groups (Table 2), and most (92%) remained in the study for the entire 12 weeks. Three refused participation (JP: n = 2; placebo: n = 1), 3 had disease recurrence (JP: n = 2; placebo: n = 1), 1 expired (placebo), and 4 were lost to follow-up (JP and placebo: n = 2 each). Overall, self-reported participant compliance was 94% on both JP and placebo.

### Serum Micronutrient Levels

A significant increase in serum  $\alpha$ -carotene ( $P = .004$ ),  $\beta$ -carotene ( $P < .0001$ ), lutein ( $P = .0004$ ), retinol ( $P = .045$ ), and  $\alpha$ -tocopherol ( $P = .023$ ) and a decrease in serum  $\gamma$ -tocopherol ( $P = .04$ ) was observed after 12 weeks of JP. No significant change was observed in tocopherols or carotenoids (except lycopene;  $P = .019$ ) in the placebo group. Statistically significant between-group differences were observed (in the change from baseline to the end of 12 weeks; Table 3) in serum  $\alpha$ -carotene ( $P = .009$ ),  $\beta$ -carotene ( $P < .0001$ ), lutein ( $P = .003$ ), and  $\gamma$ -tocopherol ( $P = .015$ ).

### P27 and Ki-67 Expression Levels

Baseline raw and posttreatment LS means for p27 and Ki-67 are shown in Table 4. The estimated treatment effect (LS mean for JP – LS mean for placebo) for p27 was 2.49 (95% CI = -1.63 to 6.61;  $P = .23$ ) and for Ki-67 was 0.14 (95% CI = 4.02 to 4.30;  $P = .95$ ). Results were similar after adjusting for covariates. There were no significant pairwise interactions with arm. None of the covariates were significantly associated with the change in p27. Only PS was significantly associated with the change in Ki-67 ( $P = .037$ ). Those with worse PS had lower posttreatment Ki-67 levels.

### Clinical Outcomes at 5-Year Follow-up

All participants were given the opportunity to receive JP for 5 years following the end of the intervention period. Although 86 of 111 participants with posttrial follow-up data chose to receive JP, not all participants took JP for the entire follow-up period. JP use was reported in approximately 67% of the follow-up visits. A total of 19 participants had SPTs, 16 occurring in the follow-up period. JP use was associated with a lower risk of a SPT, although this effect was not statistically significant (hazard ratio [HR] = 0.56; 95% CI = 0.20 to 1.57;  $P = .27$ ). After adjusting for patient characteristics, the HR was 0.88 (95% CI = 0.25 to 3.11).

### Adverse Effects/Toxicities

Although 7 adverse events were reported (JP = 3; placebo = 4), none were related to JP. Queried toxicities included

**Table 3.** Serum Micronutrient Levels Pretreatment and Posttreatment (Matched Pairs).

	Juice PLUS+				Placebo				Between Groups P Value		
	n	Baseline, Mean ± SD	12 Weeks, Mean ± SD	Change, Mean ± SD	P Value	n	Baseline, Mean ± SD	12 Weeks, Mean ± SD		Change, Mean ± SD	P Value
α-Carotene (µg/L)	52	103 ± 43.8	117 ± 54.1	13.5 ± 31.8	.004	45	117 ± 97.7	115 ± 89.8	-1.59 ± 23.7	.656	.009
β-Carotene (µg/L)	52	484 ± 414	1900 ± 1950	1420 ± 1870	<.0001	45	504 ± 577	549 ± 741	45.5 ± 209	.151	<.0001
β-Cryptoxanthin (µg/L)	52	121 ± 100	145 ± 126	24.5 ± 117	.136	45	127 ± 79.3	126 ± 70.4	-1.28 ± 29.9	.776	.130
Lutein (µg/L)	52	299 ± 111	349 ± 133	50.3 ± 95.4	.0004	45	362 ± 130	361 ± 126	-1.43 ± 71.3	.893	.003
Lycopene (µg/L)	52	558 ± 309	901 ± 1410	343 ± 1400	.084	45	549 ± 297	622 ± 307	72.4 ± 199	.019	.175
Retinol (µg/L)	51	3150 ± 923	3370 ± 905	220 ± 765	.045	45	3370 ± 898	3410 ± 868	44.7 ± 541	.582	.194
α-Tocopherol (mg/L)	51	10300 ± 3810	11700 ± 4440	1340 ± 4100	.023	45	10900 ± 3850	11200 ± 3100	247 ± 2840	.564	.128
γ-Tocopherol (mg/L)	51	3900 ± 1690	3470 ± 1120	-432 ± 1490	.043	45	3680 ± 995	3870 ± 1190	186 ± 904	.174	.015

**Table 4.** Changes in Oral Biopsy p27 and Ki-67 Expression.

Baseline	Juice PLUS+			Placebo			P Value <sup>a</sup>
	n	Mean	SD	n	Mean	SD	
Percentage p27 expression	54	18.6	11.2	45	15.1	9.54	.10
Percentage Ki-67 expression	45	26.8	8.03	42	26.2	8.31	.75
12 Weeks	n	LS Mean	SE	n	LS Mean	SE	P Value <sup>a</sup>
Percentage p27 expression	57	17.0	1.39	45	14.5	1.57	.23
Percentage Ki-67 expression	47	27.1	1.43	40	27.0	1.55	.95

<sup>a</sup>t-test at baseline; mixed-effects model at 12 weeks.

anorexia, nausea, vomiting, diarrhea, fatigue, fever, and heartburn. Heartburn occurred most commonly (13%) but was mild in all but 2 patients. Fatigue was the only toxicity with severity grade 3 (n = 1; placebo group). All other toxicities occurred in <7% of the participants, with no significant differences between groups.

## Discussion

The current study evaluated the effect of JP, a FV concentrate, on the expression of p27 and Ki-67 and the development of SPTs in patients with previous HNC. We found no significant group differences between p27, Ki-67, and any of the carotenoids or tocopherols between the JP and placebo groups. An association has been reported between tocopherols, vitamin A, and Ki-67 proliferation. Hittelman et al<sup>40</sup> reported a significant decrease in Ki-67 proliferation after administration of 1 mg/kg 13-cis-retinoic acid (a biologically active vitamin A metabolite) and 1200 IU  $\alpha$ -tocopherol ( $P = .04$ ) in the bronchial epithelium of former smokers. Participants in our study received 500% of vitamin A as  $\beta$ -carotene and 300% (4500 mg/6600 IU) of vitamin E (primarily as  $\alpha$ -tocopherol), without an effect on the Ki-67 proliferation rate.

We observed significant increase in the serum concentrations of  $\alpha$ -tocopherol and several carotenoids ( $\alpha$ - and  $\beta$ -carotene, lutein, retinol) but not  $\beta$ -cryptoxanthin or lycopene in the JP group. It is unclear how much  $\beta$ -cryptoxanthin and lycopene were present in JP because the exact amount was not quantified. We observed a significant decrease in  $\gamma$ -tocopherol (Table 3) after 12 weeks of JP. Despite vitamin E intake at 150% of daily value (22.50 mg vs recommended dietary allowance of 15 mg/d) and the abundance of  $\gamma$ -tocopherol in the American diet, lower levels of  $\gamma$ -tocopherol may reflect added  $\alpha$ -tocopherol in JP and/or increased metabolism of  $\gamma$ -tocopherol. High doses of  $\alpha$ -tocopherol may interfere with the blood and tissue concentration of other tocopherols such as  $\gamma$ - and  $\delta$ -tocopherol.<sup>41,42</sup> Additionally, smoking and systemic inflammation may also affect tocopherol metabolism.<sup>41</sup> Even though we did not

measure serum inflammatory markers, we anticipate high systemic inflammation in our participants because almost one-fourth continued to smoke (Table 2). Additionally, despite increased circulating carotenoids and  $\alpha$ -tocopherol, JP use did not have a significant effect on the incidence of SPTs in the 5-year follow-up period.

To our knowledge, this is the first randomized, double-blind, placebo-controlled trial to investigate the effect of JP on the expression of p27 and Ki-67. We also report on an observational 5-year follow-up of clinical outcomes in patients with previous HNC, some of whom took JP. Although our findings for long-term JP use and clinical outcomes were not significant, JP use during and/or after the initial trial was associated with a slightly lower rate for SPTs (HR = 0.88 after adjustment for covariates). Adding to the strength of the study design, we also assessed the serum concentration of several carotenoids and tocopherols that have previously been shown to reduce cancer risk in clinical trials. Overall, 92% of the patients completed the study, and we observed a high patient-reported compliance (94%) in both the JP and placebo groups. These relatively high completion and compliance rates may be attributed to the run-in procedures instituted prior to participant randomizations (only participants with >75% compliance were randomized into study groups).

Limitations of this study include lost samples as a result of power outage (Hurricane Katrina) and samples with bad sections, resulting in usable posttreatment p27 data for 102 participants instead of the 120 planned and lower power than anticipated. However, the LS estimate of the treatment effect was less than half of that anticipated, so it is unlikely that the extra samples would have made a meaningful difference. It is possible that the 12-week supplementation period was too short to see an impact on the SEBs. It is plausible that increased inflammation and metabolic aberrations significantly increase nutrient needs in HNC patients, which were insufficiently met by JP and, thus, was unsuccessful in decreasing SEBs associated with SPTs. Moreover, continued tobacco and alcohol use increase oxidative stress, further compounding the problem. The results of this study

may also not be generalizable to other cancers because HNC patients are at a higher risk for developing SPTs. Finally, although many participants agreed to consume JP after the completion of the clinical trial, JP use during this follow-up period was not randomized, creating limitations in the usefulness of the data and in the interpretation of results.

## Conclusions

In conclusion, 4 capsules of JP consumed daily for 12 weeks increased serum levels of most carotenoids and  $\alpha$ -tocopherol but failed to modulate the expression of p27 and/or Ki-67 levels in HNC patients. We also did not observe any effects of age, tumor characteristics (ie, site and stage), and continued tobacco and/or alcohol use on p27, Ki-67, or SPTs in these patients. Longer use of JP also did not appear to be associated with SPTs. Future studies should evaluate longer supplementation period, modified formulations of supplements with demonstrated chemopreventive properties in preclinical models, nutritional status, and the role of oxidative stress and inflammatory markers on SPT in this population.

## Authors' Note

Clinical trial registration number: NCT00064298. <https://clinicaltrials.gov/ct2/show/NCT00064298?term=%22juice+plus%22+AND+%22head+and+neck+cancer%22&rank=1>.

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