

**Comprehensive Cancer Center of Wake Forest University -- CCOP RESEARCH BASE-CCCWFU #60A02  
A Phase II Randomized Placebo Controlled, Double Blinded Trial to Evaluate the Effects of Fruit and Vegetable  
Extracts on Intermediate Biomarkers in Head and Neck Cancer Patients**

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## 1.0 Background and Rationale

### 1.1 Head and Neck Squamous Cell Carcinoma (HNSCC)

In 2003, about 27,700 and 9,500 Americans will be diagnosed with oral cavity/pharynx and larynx cancers, respectively, and 11,100 patients will die of these cancers (ACS, 2003). African-American patients have poorer survival outcomes after treatment for oral and pharyngeal cancer, compared with their white counterparts (Moore *et al.*, 2001). Despite advances in treatment, long-term survival has remained at less than 50% over the past 40 years (Boring *et al.*, 1994). HNSCC often develops as multiple lesions throughout the mucosa of the upper aerodigestive tract. While the primary cancers have been successfully treated, the development of second primary tumors (SPTs) is the most important factor determining survival of early-stage HNSCC patients. A 3-7% SPT rate per year has been reported (Day and Blot, 1992).

In a study of 851 patients with initial squamous cell carcinoma of the larynx, tonsils, pyriform sinus, oral cavity, oral tongue, and base of tongue, the probability of developing a SPT 5-years after undergoing treatment for the initial head and neck cancer was 22% (Schwartz *et al.*, 1994). Patients with oral cavity and laryngeal primary tumors also have a 30% and 76% incidence, respectively, of second primaries located in the lung (Licciardello *et al.*, 1989). Three risk factors for SPTs are: (1) the site of the initial primary cancer, (2) tobacco smoking, and (3) alcohol consumption (Schwartz *et al.*, 1994). A recent study indicates significantly higher smoking-related SPT rates in active smokers versus those who have never smoked, with intermediate rates for former smokers (Khuri *et al.*, 2001). The origin of SPTs seems to be influenced by the field cancerization effect and an increased baseline genetic predisposition. Studies of chemoprevention in the lungs and upper aerodigestive tract have relied on the field carcinogenesis hypothesis, which predicts that diffuse epithelial injury will result from a general exposure of target tissues to carcinogens (Lippman *et al.*, 1994). The hypothesis of field cancerization is supported by the frequent occurrence of multiple primary tumors within the exposed field (Schwartz *et al.*, 1994). Squamous cell carcinoma of the head and neck serves as an ideal model for the development of chemopreventive strategies (Kim *et al.*, 2002).

### 1.2 Chemoprevention of HNSCC

To improve HNSCC patients' survival, an effective program of chemoprevention for second malignancies is essential. While reduction of cancer risk is associated with smoking cessation, the elevated risk may persist 5 to 6 years after smoking cessation (Spitz *et al.*, 1988). Several clinical trials failed to show beneficial effects of beta-carotene, vitamin E, 13-cis-retinoid acid combined with alpha-tocopherol and INF-alpha, vitamin A, and/or *N*-acetylcysteine in reverting advanced pre-malignant lesions or preventing SPTs of the upper aerodigestive tract (Stich *et al.*, 1988; Lippman *et al.*, 1993; Benner *et al.*, 1993; van Zandwijk *et al.*, 2000; Papadimitrakopoulou and Hong, 2000; Mayne *et al.*, 2001). Questions over whether the observed association between HNSCC cancer risk and serum carotenoids is related to F&V (Fruit and Vegetable) intake or whether carotenoids may simply be markers of F&V consumption that have little or no chemopreventive value have been raised (Collins *et al.*, 1998). It is also possible that a single chemopreventive agent cannot provide the overall beneficial effect of the various chemopreventive compounds combined in F&V. In addition, the widespread use of retinoic acid as a chemopreventive agent has been limited by the toxic side effects experienced by individuals who are on the drug for prolonged periods of time, favoring less harmful alternative approaches like the F&V supplements proposed in this research. Lastly, the traditional research strategy, conducting intervention trials using one substance at one dose or various combinations of substances, could take several decades to discover an effective chemopreventive substance in humans. This is true especially if there is no "magic bullet" in F&V, but it is the combination of agents, each of which is needed for optimal or synergistic effects that is

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important. F&V extract supplementation may provide effective long-term chemoprevention, without side effects, for both high-risk cancer patients and healthy populations.

### 1.3 Dietary Modulation vs. F&V Supplements in Cancer Prevention

Based on the fact that the main risk factors for HNSCC are smoking and alcohol consumption as well as inadequate F&V consumption, intervention should focus on smoking cessation, decreased alcohol use, and increased F&V intake (Winn *et al.*, 1984; Tavani *et al.*, 1994; Pawlega, 1998). However, lifestyle and behavior change may not be easy for HNSCC patients. For example, it is a common observation that a substantial proportion of HNSCC patients stop smoking after diagnosis but resume after treatments (Christensen *et al.*, 1999). Many patients also continue to consume alcohol (Christensen *et al.*, 1999). An important advantage of using F&V extract supplementation is that we are able to deliver a relatively consistent combination of test agents. In contrast, study subjects instructed to increase F&V intakes may have different preferences. Since F&V differ widely in their nutrient contents, we propose to use a commercially available product that contains a known mixture of F&V with potential chemopreventive agents. A placebo of similar taste and appearance will be formulated for comparison.

### 1.4 Molecular Mechanisms of F&V in Cancer Prevention

We believe that it is extremely important to systematically test the efficacy and safety of food-derived agents before we recommend them for widespread, long-term use in cancer patients and general populations. Many food-derived agents are extracts, containing multiple compounds or classes of compounds. Some of the potential chemopreventive agents found in the F&V extracts include: quercetin and vitamin E (apples, cabbage, and kale), vitamin C (citrus fruits), sulforaphane (broccoli), flavonoids (cranberries), beta-carotene (carrots), and lycopene (tomatoes) (Hollman *et al.*, 1997; Steinmetz and Potter, 1996; Fahey *et al.*, 1997; Wilson *et al.*, 1998; Sies and Stahl, 1998; Beecher, 1998; Krinsky, 1998). Many of these compounds have unique chemopreventive properties, and several examples are described below. Data from both animal and epidemiological studies suggest that dietary antioxidants may have protective effects against certain types of cancer. Specifically, vitamin E, a well-studied, lipid-soluble antioxidant, was associated with a reduced risk of cancer in two longitudinal epidemiological studies (Knekt *et al.*, 1988; 1991). The potential use of vitamin E in human cancer chemoprevention has been evaluated in lung cancer (alpha-Tocopherol, Beta-carotene Cancer [ATBC] Prevention Study Group, 1994), oral leukoplakia (Benner *et al.*, 1994), and colorectal polyps (McKeown-Eyssen *et al.*, 1988; deCosse *et al.*, 1989). In the ATBC Study cohort, higher serum-tocopherol status is associated with lower lung cancer risk; this relationship appears stronger among younger persons (<60 years) and those with less cumulative smoke exposure (< 40 years of smoking) (Woodson *et al.*, 1999).

The anti-mutagenic and anti-proliferative effects of alpha-tocopherol may contribute to its chemopreventive property (Kelloff *et al.*, 1994). Antioxidant activity against different reactive oxygen species (ROS) was evaluated in juice from different fruits (Wang *et al.*, 2000). Blackberry, strawberry, cranberry, raspberry, and blueberry had the highest antioxidant capacity against superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. In a study with tomato juice, carrot juice, and dried spinach powder, oxidative base damage was significantly reduced during the carrot juice intervention (Pool-Zobel *et al.*, 1997). In addition, using a method for the determination of the carotenoid content in selected vegetables, it was found that good sources for lutein are spinach, kale, and broccoli, and sources for beta-carotene are broccoli, spinach, kale, carrots, and tomatoes (Huck *et al.*, 2000). Dietary phenolic compounds, ubiquitous in F&V and their juices, possess an antioxidant

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activity that may benefit human health. In an *in-vitro* study, both fresh and commercial apple juices inhibited copper-catalyzed low-density lipoprotein (LDL) oxidation. The data support the inclusion of apple and its juice in a healthy human diet (Pearson *et al.*, 1999). Peach

flesh extract, whole peach extract, and peel extract also inhibit LDL oxidation (Chang *et al.*, 2000). Flavonoids display a wide range of pharmacological properties including anti-inflammatory, anti-mutagenic, anti-carcinogenic and anti-cancer effects. Previous research has suggested that the potential anti-tumor properties of quercetin (QC) (apples, cabbage, kale) include immune stimulation, free radical scavenging, alteration of the mitotic cycle in tumor cells, gene expression modification, anti-angiogenesis activity, or apoptosis induction, or a combination of these effects (Hayashi *et al.*, 2000). Recent evidence demonstrates that oral administration of QC and pH-modified citrus pectin (MCP; a polysaccharide found in the cell wall of plants) can reduce the growth of primary solid tumors in an animal model (Hayashi *et al.*, 2000). QC effectively inhibits a wide range of protein kinases, including the epidermal growth factor receptor (EGFR) tyrosine kinase, which may have potential as anti-cancer and anti-metastasis agents (Huang *et al.*, 1999). In laryngeal cancer cells, QC exerts a dose-dependent growth-inhibitory effect that was associated with a block of the cells at the G<sub>2</sub>/M checkpoint of the cell cycle followed by apoptosis (Ferrandina *et al.*, 1998). The data suggest that QC may have potential application in laryngeal cancer treatment.

Epidemiological data suggest a cancer-preventive effect of brassica vegetables, including all types of cabbages, broccoli, cauliflower, and brussels sprouts. They may protect against cancer due to their glucosinolate content. Some of the hydrolysis products, such as indoles and isothiocyanates, influence phase I and phase II drug metabolism enzymes, thereby possibly influencing carcinogenesis (van Poppel *et al.*, 1999). Sulforaphane (SF) is one of the dietary isothiocyanates (ITCs) that are abundant in foods derived from vegetables, and many ITCs are potent cancer chemoprotective agents in animal systems (Zhang 2001). Many ITCs rapidly accumulate to very high concentrations in cells, playing a critical role in inducing anticarcinogenic phase II drug detoxification enzymes. In cell culture systems, SF inhibits the formation of DNA adducts following exposure to benzo[a]pyrene (BP) and 1,6-dinitropyrene (1,6-DNP), probably by stimulating the expression of glutathione-S- transferase (GST) and AD(P)H-quinone reductase (Singletary and MacDonald, 2000). Two potential targets of phenolic antioxidants and natural products (flavonoids and isothiocyanates) have been proposed: the mitogen-activated protein kinases (MAPKs) and the ICE/Ced-3 proteases (caspases) stimulated by these agents (Kong *et al.*, 2000).

In summary, many of the active compounds in F&V extracts have been studied, and the mechanistic and pharmacological data can be used to characterize the chemopreventive potential of the extracts. Many of these compounds play critical roles in blocking either the carcinogenesis pathway or tumor growth, both of which are critical in preventing the conversion of preneoplastic lesions to tumor or SPTs.

### 1.5 Surrogate Endpoint Biomarkers (SEBs) in Chemoprevention Trials

Carcinogenesis is a multistep process, and the driving force behind it is thought to be genetic damage caused by continuous exposure to carcinogens, deficient detoxification pathways, and defective DNA repair. These genetic alterations, combined with other risk factors, may lead to phenotypic changes in target tissues; i.e., dysregulation of cell proliferation, abnormal DNA ploidy and nuclear pleomorphism, and differentiation. Chemoprevention aims to reverse this process by regaining normal regulation of cell proliferation and differentiation and eliminating genetically and phenotypically aberrant cells. HNSCC provide an excellent model for chemoprevention based on three features: exogenous exposure to tobacco and alcohol are the major risk factors; carcinogenesis proceeds in multiple steps and throughout the field. Most of the genetic alterations and phenotypic abnormalities associated with the histologic transitions from normal epithelium to hyperplasia to metaplasia,

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dysplasia, and malignancy have great potential as intermediate endpoints for predicting risk for secondary tumors and monitoring the outcome of chemopreventive trials. The application of such biomarkers will significantly shorten the time required to evaluate the clinical efficacy of promising chemopreventive agents. Based on these premises, two types of promising SEBs are summarized below.

Prognostic Markers: Several studies have shown that a low p27 and/or high Ki-67 labeling index correlated with tumor progression and poor prognosis for patients with head and neck cancers (Saito *et al.*, 1999; Xie *et al.*, 1999; Mineta *et al.*, 1999; Kudo *et al.*, 2000). P27, a member of the cip/kip family of cyclin-dependent kinase inhibitors, plays a critical role in cell cycle regulation from the G<sub>1</sub> to S phase (Sherr, 2000). The Ki-67 protein is a nuclear and nucleolar protein, tightly associated with somatic cell proliferation (Endl and Gerdes, 2000). Smoking appears to elicit dose-related higher Ki-67-labeling indices in the bronchial epithelia of active smokers (Lee *et al.*, 2001). Although the proliferative response decreased gradually in former smokers, a subset of individuals had detectable proliferation for many years. Our working hypothesis is that modulation of p27 and/or Ki-67 expression levels may influence recurrence and overall survival. The ultimate objective of chemoprevention in patients with previous HNSCC is to halt the growth of premalignant lesions and further progression.

Cancer Risk Markers: Lipid peroxidation and DNA damage were significantly higher in HNSCC patients (Seven *et al.*, 1999; Yu *et al.*, 1999; Cloos *et al.*, 2000; Schmezer *et al.*, 2000). Smoking and excess alcohol consumption contribute to oxidative stress and risk for HNSCC (Schwartz *et al.*, 1994). In animal models and humans, ethanol consumption increased levels of the lipid peroxidation product malondialdehyde (MDA) and DNA damage (Mufti *et al.*, 1997; Maffei *et al.*, 2000; Mutlu-Turkoglu *et al.*, 2000). In addition, HNSCC patients have profound defects in their immune defenses, which are associated with disease stage and prognosis (Heimdal *et al.*, 1998; Heimdal *et al.*, 1999). In two previous studies of healthy volunteers, F&V extracts were found to decrease DNA damage, lower lipid peroxide levels, and boost immune response (Thompson *et al.*, 1999; Smith *et al.*, 1999). Thus, our working hypothesis is that F&V decrease DNA damage, boost immune response, and prevent SPTs in HNSCC patients.

### 1.6 Effects of F&V Extracts on SEBs

In three studies of healthy subjects, supplementation with F&V extracts (Juice Plus™) for 28-80 days significantly increased serum antioxidants: alpha-carotene and beta-carotene (Wise *et al.*, 1996; Inserra *et al.*, 1999; Smith *et al.*, 1999) (Table 1). Two studies also showed a significant increase in lutein/zeaxanthin, lycopene, and alpha-tocopherol (Wise *et al.*, 1996; Inserra *et al.*, 1999). Clinical trial results showed that immune function was significantly enhanced by F&V extracts measured as proliferation of peripheral mononuclear leukocytes (PMLs) (about 2-fold increase), natural killer (NK) cell cytotoxicity (about 1.4-fold increase), and interleukin-2 (IL-2) production by PML stimulated with phytohemagglutinin (PHA) (about 5-fold increase) (Inserra *et al.*, 1999). A statistically significant increase in IL-2 production was also observed in smokers. In another clinical trial, DNA damage, measured as the mean comet tail moment, decreased from 13.24 (SD: 2.77) to 4.41 (SD: 2.76) 80 days after treatment with F&V extracts (Smith *et al.*, 1999). Similar beneficial effects were observed in both smokers and non-smokers. In summary, many of the compounds in F&V extracts play critical roles in either blocking carcinogenesis pathways or tumor growth, which may prevent the conversion of preneoplastic lesions to tumors or SPTs.

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**Table 1. Effects of F&V Extracts on Serum Antioxidant Levels**

Antioxidant (µg/ml)	Wise <i>et al.</i> , 1996 (n=15)		Inserra <i>et al.</i> , 1999 (n=46)		Smith <i>et al.</i> , 1999 (n=20)	
	Baseline	Day 28	Baseline	Day 80	Baseline	Day 80
Lutein/Zeaxanthin	0.36±0.16 <sup>1</sup>	0.52±0.17*	0.21±0.08 <sup>1</sup>	0.28±0.12*	0.23±0.09	0.29±0.12
β-Cryptoxanthin	ND	ND	0.08±0.05	0.09±0.05	0.09±0.05	0.09±0.05
Lycopene	0.14±0.17	2.94±3.27*	0.30±0.14	0.35±0.14*	0.31±0.15	0.346±0.14
α-Carotene	0.04±0.04	0.08±0.06*	0.07±0.04	0.28±0.13*	0.07±0.04	0.27±0.1*
β-Carotene	0.1±0.1	0.43±0.34*	0.33±0.03	0.88±0.06*	0.38±0.04	0.84±0.05*
α-Tocopherol	8.9±2.3	14±4.2*	22.6±8.5	28.8±9.8*	25.8±9.1	30.4±9.5

<sup>1</sup> Data are presented as mean±SD; ND, not determined

\* p<0.05, post treatment vs. baseline

### 1.7 Effects of Depression on Immune Function and DNA Damage

Smoking and alcohol consumption in head and neck cancer patients and its treatment can have important psychosocial implications and many patients are depressed (de Leeuw *et al.*, 2001; Duffy *et al.*, 2002). Depression has been associated with mortality but the mechanism is still unclear. Previous studies have demonstrated that higher stress levels are associated with immunosuppression and elevated DNA damage (Maes *et al.*, 1999; Irie *et al.*, 2001; Nunes *et al.*, 2002). Therefore, we decided to also measure stress levels with a short perceived stress scale to test the working hypothesis that depression and stress can decrease immune function, increase DNA damage, and result in tumor progression. As shown in a study of 362 healthy workers, female subjects showed positive relationships between oxidative DNA damage and the tension-anxiety, depression-rejection, anger-hostility, fatigue, and confusion scores of the profile of mood states, respectively (Irie *et al.*, 2001). An association between depression and altered immune and hormonal systems has been suggested by the results of many studies (Maes *et al.*, 1999; Saha *et al.*, 2001; Scanlan *et al.*, 2001; McGuire *et al.*, 2002; Nunes *et al.*, 2002). We will collect information on depressive symptoms and stress levels pre and post intervention using two validated questionnaires with good psychometric properties, Center for Epidemiologic Studies Depression Scale (CES-D) and Perceived Stress Scale (PSS). We will evaluate whether depressive symptoms and stress levels change as well as what the absolute stress and depression levels are. This information will lead to future research to treat depression, improve immune function, decrease DNA damage, and decrease risk for second primary tumors.

### 1.8 Effects of Smoking on Circulating Antioxidant Levels

In addition to many known carcinogens present in cigarette smoke, it is also a source of oxidative stress. Therefore, it is important to understand the relationship between cigarette smoking and circulating concentrations of antioxidant micronutrients. Several previous studies reported that free radicals in cigarette smoke may deplete plasma antioxidants in vitro and lower plasma antioxidant concentrations in smokers in vivo (Frei *et al.*, 1991; Eiserich *et al.*, 1995; Marangon *et al.*, 1998; Lykkesfeldt *et al.*, 2000). However, it has been difficult to determine whether differences in plasma antioxidants between smokers and nonsmokers are actually due to the effect of cigarette smoking exposure or are due to differences in dietary antioxidant intakes or other covariates. For example, since dietary micronutrient intake is correlated with circulating micronutrient concentrations, consumption of fruits and/or vegetables has been correlated with blood concentrations of vitamin C, total carotenoids, α-carotene, β-carotene, β-cryptoxanthin, and lutein/zeaxanthin (review by Alberg, 2002). Smokers usually have lower circulating concentrations of antioxidant micronutrients based on the simple fact that cigarette smokers tend to eat less healthful diets in general than nonsmokers and specifically to consume less F&V (Hebert and Kabat, 1990; Larkin *et al.*, 1990; Morabia and Wynder,

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1990; Marangon *et al.*, 1998). However, the results from a recent study indicated that active smoking influenced plasma concentrations of total ascorbic acid, several carotenoids, and  $\gamma$ -tocopherol -after control for dietary antioxidant intakes, F&V intakes, body mass index, and other covariates (Dietrich *et al.*, 2003). Furthermore, they also measured plasma cotinine concentrations to avoid possible misclassification due to the use of self-reported smoking status. In summary, the differences of circulating antioxidant micronutrient concentrations observed between smokers and nonsmokers seem to be mainly due to an acute effect of smoking after adjustment for dietary antioxidant micronutrient intake and other potential confounding factors. Therefore, it's important to monitor dietary antioxidant intake, smoking activity, and other covariates during chemoprevention trial.

## **2.0 Objectives**

The proposed research will use a randomized, double-blind, placebo-controlled trial to evaluate the effects of fruit and vegetable (F&V) extracts on surrogate endpoint biomarkers (SEBs) that are associated with the development of SPTs in patients with previous HNSCC.

- 2.1 To test the hypothesis that F&V extracts can modulate SEBs in patients with previous HNSCC. The primary endpoint is the expression of a cell cycle regulatory protein, p27, which is associated with disease-free survival. The secondary endpoints are cell proliferation (Ki-67), DNA damage (strand breaks), and immune function (T-cell function), which are associated with the development of HNSCC.
- 2.2 To evaluate whether the augmentation of SEBs by F&V extracts is influenced by other factors, such as characteristics of the original tumor (i.e., site and stage), continued tobacco/alcohol use, or depression. Our working hypothesis is that current tobacco/alcohol use and/or depression may modify the effects of F&V extracts on SEBs. Therefore, tobacco/alcohol use and depression will be evaluated at baseline and post treatment.
- 2.3 To determine serum carotenoids and antioxidant levels (vitamins A, C, and E) at baseline and post treatment. This evaluation tests the hypothesis that F&V extracts can increase plasma antioxidant levels which may contribute to biomarker changes. We will also assess whether the augmentation of plasma antioxidant levels is influenced by tobacco use and/or alcohol consumption.

The proposed research will use a novel complementary and alternative (CAM) approach to cancer chemoprevention. The results will provide critical information on: (1) the use of surrogate endpoint(s) in monitoring the biological effects of F&V extracts, (2) the mechanisms of F&V in preventing HNSCC, and (3) the feasibility of using supplementation of F&V extract supplementation with HNSCC patients. Promising study results from this Phase II chemoprevention trial will lay the groundwork for a larger Phase III trial to assess the application of F&V extracts in preventing SPTs for patients with HNSCC.

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**3.0 Patient Selection Criteria**

3.1 Eligibility

- Signed protocol specific informed consent prior to registration/randomization
- Curatively treated squamous cell carcinoma of the upper aerodigestive tract; Primary sites include only those regions within the oral cavity, oropharynx, hypopharynx, and larynx
- Primary lesions must have been Stage I, II, III or IVa (American Joint Committee on Cancer, 1988); free of head and neck cancers for a minimum 6 months, up to a maximum of 3 years following completion of surgery, radiation therapy, and/or chemotherapy; No synchronous tumors
- Age  $\geq$  18
- Performance status  $\geq$  70% (0 to 1 by ECOG/Zubrod Scale)
  
- Normal hematological parameters (hemoglobin  $>$  10g/dl; WBC  $\geq$  3,000; platelets  $\geq$  100,000), adequate liver function (bilirubin  $\leq$  1.5 mg/dl, SGOT $<$ 40, SGPT $\leq$  56), adequate renal function (creatinine  $\leq$  1.5 mg%)
- Life expectancy:  $\geq$  6 months
- Negative serum pregnancy test within 10 days prior to registration
- Prior treatment with: (must be  $>$ 6 months and  $<$ 3 years prior to study entry)  
Radiation                    Acceptable:   X   Yes           No  
Study Agents            Acceptable:   X   Yes           No  
Chemotherapy           Acceptable:   X   Yes           No  
Hormonal Therapy        Acceptable:   X   Yes           No

3.2 Ineligibility

- Concomitant malignancy other than curatively treated HNSCC within last 5 years, except non-melanoma skin cancer and insitu carcinoma of the cervix
- Serious medical or psychiatric illness which would prevent informed consent
- Expected survival  $<$  6 months
- Nausea  $\geq$  grade 2 by NCI Common Toxicity Criteria (CTC) Version 3.0
- Current supplementation of high-dose vitamins within the last 2 months (10 times the recommended daily allowance (RDA): vitamin A, 8,000-10,000 IU; vitamin C, 600 mg; and vitamin E, 80-100 IU).

**4.0 Treatment Plan**

4.1 Study Design

This study is a two arm, placebo controlled double-blind, Phase II chemoprevention trial assessing the effects of F&V extracts on biomarkers in 200 patients with previous HNSCC.

4.2 Treatment Schedule

A research nurse will conduct a screening interview to collect information on medical history, tobacco use, and alcohol consumption history and to administer a food frequency questionnaire. After the interview, the research nurse will collect approximately 1 ½ tablespoons of blood for screening evaluation. Patients with normal blood chemistry will be invited to initiate the 1-week

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run-in procedure. Study subjects with  $\geq 75\%$  compliance will be randomly assigned to receive either F&V extracts or placebo.

Participants in the treatment group will receive two fruit and vegetable capsules in the morning and two fruit and vegetable capsules in the late afternoon or very early evening each day; participants in the placebo arm will receive two placebo capsules in the morning and two placebo capsules in the late afternoon or very early evening each day. The study plane includes two blood draws and two biopsies, one at baseline and one at 12-week after intervention. Supplements will be stopped after the 12-week treatment period. Patients will be given a \$10 gift certificate after baseline visit and a \$40 gift certificate when they complete the study. Patients may have treatment discontinued for any of the following reasons: (1) histological progression, either as dysplasia or invasive carcinoma; (2) development of unacceptable gastrointestinal (GI) toxicity ( $\geq$  grade 3); (3) illness necessitating premature termination; (4) protocol violation; and (5) death. The investigator will make every effort to perform the final evaluation at 12 weeks.

#### 4.3 Concomitant Treatment

No concomitant cancer treatment (i.e., surgery, radiation therapy, or chemotherapy) or chemopreventive agents is acceptable. Study subjects will be instructed to maintain their usual dietary habit and lifestyle during the trial.

#### 4.4 Post-Treatment Use of Fruit and Vegetable Extracts

Patients will be given the option to continue taking Juice Plus free of charge for five years, following completion of the 12-week study.

### 5.0 Pharmaceutical Information

The test supplements consist of dried F&V powders. Fruit juice extracts from apples, oranges, pineapples, papaya, cranberries, and peaches and vegetable juice extracts from carrots, parsley, beets, broccoli, kale, cabbage, spinach, and tomatoes are cryoevaporated (Juice Plus™, NSA International, Memphis, Tennessee) to concentrate and preserve nutrients, particularly carotenoids. The blends are encapsulated in hard gelatin capsules to provide 850 mg of fruit powder per fruit capsule and 750 mg of vegetable powder per vegetable capsule. Test supplements will be from the same lot. F&V extracts should be considered as a GRAS (Generally Recognized As Safe) product. In several clinical trials using F&V extracts as dietary supplements with healthy subjects, the dosage proposed in this study has been tested. No toxic effect was reported (Wise *et al.*, 1996; Smith *et al.*, 1999; Inserra *et al.*, 1999). Since the extracts have not been tested in cancer patients, to be cautious, we will conduct telephone interviews at weeks 1, 2, 3, 4, and 8 weeks of treatment. A questionnaire will be used to record potential treatment-related side effects, particularly gastrointestinal (GI) toxicities, such as nausea, vomiting, fever, diarrhea, heartburn, abdominal pain, or others. Patients with severe adverse reactions ( $\geq$  grade 3) will stop the treatment. If patient chooses to continue taking Juice Plus for 5 years following completion of the 12-week study, the company will provide the Juice Plus to the patient free of charge.

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**6.0 Treatment Modifications**

6.1 Toxicity Criteria

Toxicity will be determined using the NCI Common Toxicity Criteria (CTC), version 3.0 for Toxicity and Adverse Event Reporting (CTEP home page: <http://ctep.info.nih.gov>).

6.2 Toxicity Reporting - Notify the study chairman *immediately* by telephone of an unexpected, life-threatening (grade 4) or unexpected, fatal (grade 5) adverse event with an attribution of possible, probable or definite. These events must be reported within (10) working days to DCP/NCI, FDA, and CCCWFU CCOP Research Base Data Management Center (fax: 336-716-5687). The AE should be reported on the FDA Form 3500 MedWatch (available from the FDA - [www.fda.gov/medwatch](http://www.fda.gov/medwatch).) These events should also be reported to site specific IRBs according to their individual policy.

6.3 Treatment Modification

No toxicity related to F&V extracts is expected. However, since GI toxicity is a potential concern, specific guidelines have been developed to evaluate it. The research nurse will conduct telephone interviews weekly during the first month of treatment. Subjects will be asked to rate symptoms of GI upset on a five-point scale: (0) No symptoms of heartburn or abdominal pain; (1) Occasional epigastric pain (heartburn) of mild intensity, relieved by medication; (2) moderate epigastric pain (heartburn) one to two days per week, usually relieved by medication; (3) Moderate pain > two days per week, not relieved by medication; and (4) Severe pain that wakes subject from sleep, not relieved by medication. Subjects who experience equal to, or greater than, Grade 3 (using the CTC, version 3.0) symptoms will stop the treatment.

**7.0. Response Evaluation Criteria**

Response to treatment will be determined by comparing SEBs, using oral biopsies (i.e., p27 and Ki-67 labeling index) and blood samples (i.e., DNA damage and immune function) taken at baseline and at the end of the 12-week treatment period.

**8.0 Study Parameters**

- 8.1 Screening: medical history/pathology reports; physical examination; performance status; hematology and blood chemistry profile, and questionnaires
- 8.2 Run-In: medication diary and pill count (placebo only)
- 8.3 Baseline: biopsies and blood samples for biomarkers; baseline toxicity.
- 8.4 Interim evaluation: GI Toxicity Telephone Interview (weeks 1, 2, 3, 4, and 8); medication diary and pill count at Run-in/baseline and 12 week.
- 8.5 Post-Treatment: questionnaires; biopsies and blood samples for biomarkers; toxicity.
- 8.6 Study parameters: as shown in Table 2.

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**Table 2. Study Parameters**

	*Screening	Run-In/ Baseline	1	(Week)					12	q year x5 off- study f/u
Medical History	X									
Signed Informed Consent	X									
Physical Exam	X							X		
Performance Status	X							X		
Hemoglobin	X							X		
WBC	X							X		
Platelets	X							X		
Bilirubin	X							X		
Creatinine	X							X		
SGOT	X							X		
SGPT	X							X		
Serum Pregnancy Test**	X							X		
Biomarkers		X						X		
Oral Biopsies		X						X		
CESD		X						X		
PSS		X						X		
Baseline H&N Questionnaire		X						X		
Food Questionnaire		X						X		
GI Toxicity Telephone Interviews			X	X	X	X	X			
Post Treatment H&N Questionnaire								X		
Medication Diary (placebo)								X		
Pill Count		X						X		
Follow-up Form									X	

\*Within 28 days of registration

\*\* Within 10 days of registration

## **9.0 Registration/Randomization and Data Management Procedures**

### **9.1 Registration, Stratification, and Randomization**

All patients entered on a CCCWFU trial must be registered with the CCCWFU Protocol Registrar at 336-716-4536 between 8:30 AM and 4:00 PM Eastern time, Monday - Friday. Patients must be randomized through the registrar prior to initiation of treatment. Patients will be stratified by tobacco use, alcohol consumption, and tumor stage at diagnosis, and randomized within strata (12 categories: tumor stage I, II, III or IVa by tobacco and alcohol) to F&V or placebo with equal probability. The CCCWFU Protocol Registration/Eligibility Form (Appendix II) The Initial Flow Sheet and signed Consent Form should be completed prior to telephone registration. The registrar will call the site personnel back with information on where to fax the registration. If the patient meets all eligibility criteria, the patient will be randomized to one of the treatment arms.

### **9.2 Test Agent Ordering**

Storage and distribution of Juice Plus™ and placebo will be handled by Biologics, Inc. (625 Oberlin Road, Raleigh, NC, 27605; 1-800-850-4306). **The CCCWFU protocol registrar will contact Biologics, Inc. at the time of patient randomization to initiate shipment of either Juice Plus™**

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**or placebo to the participants.** Each shipment will include a twelve week supply of test agent or placebo.

The contact person in Dr. Hu's laboratory is Laura Gilliam, NRC-441, Wake Forest University Health Sciences, Telephone (336) 713-7645 or (336) 713-7649.

9.3 Data Management Procedures

<u>Data</u>	<u>When Due</u>
Protocol Registration Form / Eligibility Checklist (Appendix II) Initial Flow Sheet (Appendix III) Signed Consent Form, HIPPA Form Baseline H&N Risk Questionnaire (Appendix XII) Baseline blood and initial biopsy samples (Appendix XVII)	Within 1 week of registration  Within 1 week of registration Within 1 week of registration Within 1 week of registration  At registration
GI Specific Questionnaire (Telephone Interview) (Appendix XIV) CESD (Appendix IV) PSS (Appendix V) H&N Risk Questionnaire Post-Treatment (App XV) Food Questionnaire (Appendix XIII)	One week after interviews (wk 1, 2, 3, 4, and 8)  Baseline and 12 weeks Baseline and 12 weeks 12 weeks  Baseline and 12 weeks
Flow Sheet (Appendix III)	One week after baseline and 12 week visits
Medication Diary and Pill count (App VI)	Within one week of baseline visit and week 12 visit
Off Treatment Form (Appendix XVI)	Follow yearly for 5 years
Final Biopsy specimen and Biomarkers (Appendix XVII)	At 12 week visit

**10.0 Statistical Considerations**

10.1 Objectives: The study's primary objective is to assess the effects of F&V extracts on cell cycle regulation as quantified by p27. Secondary objectives assess the effects of F&V extracts on other SEBs that quantitate DNA damages and immune function are to assess whether the effects of F&V can be influenced by other factors, such as stage/site of primary tumor, tobacco use, and alcohol consumption. Patients will be stratified by tobacco use, alcohol use, and primary tumor stage, and randomized within strata to F&V or placebo with equal probability. Analysis of primary and secondary biomarker measures will be carried out based on an "intent to treat" approach. That is, all randomized eligible patients will be used in all analyses, whether or not they are actually treated or treated appropriately.

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- 10.2 Study Design and Randomization: A prospective, randomized, double-blind Phase II design will be used to assess the cancer preventive effects of F&V on biomarkers in head and neck cancer patients. Patients will be stratified by tobacco use, alcohol use, and primary tumor stage. Within each stratum, patients will be randomized to receive F&V or placebo, using blocked randomization to ensure approximately equal accrual to each treatment throughout the study. Block sizes of varying length will be determined randomly to ensure that future assignments cannot be inferred from past assignments. Treatment assignments will be computer generated and linked to the registration system.
- 10.3 Sample Size and Feasibility: The study’s primary objective is to assess differences in p27, the biomarker quantitating cell-cycle regulation. The sample size is based on a t-test comparison between treatment groups at the 5%, two-sided level of significance. The sample size per group needed to detect a 20% relative difference between groups (assuming a mean±SD p27 value in the control group of 34.68±11.41) with 80%, 85%, and 90% power are 46, 52, and 60, respectively. These estimations are probably conservative since they do not account for the baseline adjustment that will be used in the analysis. Based on these calculations, we will need 60 evaluable in each treatment arm. Assuming a 40% dropout rate, we will need a total of 200 subjects to complete the trial. Approximately 400 potential participants are followed yearly at WFUBMC. Assuming a comparable number will be followed at our CCOP affiliates combined and that about 30% of the patients will agree to participate (total n=240), the conservative estimate is that we will be able to complete accrual (n=200) to this study in less than 18 months.
- 10.4 Power for Assessing Study Outcomes: A final sample size of 60 patients per group (out of 100 with 40% drop out rate) will allow us to detect a relative difference of 20% or less between treatment and placebo in the primary biomarker with 90% power. Table 3 shows the minimal treatment differences in both primary and secondary biomarkers that can be detected with 80% and 90% power at the 5% two-sided level of significance. Table 4 shows the minimal detectable treatment differences in plasma antioxidant levels that can be detected with 80% and 90% power at the 5% two-sided level of significance.

**Table 3. Minimal Detectable Differences in Primary and Secondary Biomarkers**

Biomarker	Mean	SD	Minimal Detectable Difference	
			Absolute (Relative %)	
			80%	90%
Cell-Cycle Regulation <sup>1</sup>	34.68 (n=94)	11.41	5.88 (17%)	6.81 (20%)
Cell Proliferation <sup>2</sup>	65.1 (n=85)	14	7.22 (11%)	8.36 (13%)
DNA Damage <sup>3</sup>	13.24 (n=20)	2.77	1.43 (11%)	1.65 (12%)
Immune Function <sup>4</sup>	3.83 (n=12)	0.47	0.24 (6%)	0.28 (7%)

<sup>1</sup> Immunohistochemistry staining of P27-positive cell rate (Mineta *et al.*, 1999)

<sup>2</sup> Immunohistochemistry staining of Ki-67-positive cell rate (Xie *et al.*, 1999)

<sup>3</sup> DNA strand breaks measured as comet tail moment (Smith *et al.*, 1999)

<sup>4</sup> T-cell response to IL-2 in head and neck cancer patients (Pandit *et al.*, 2000)

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**Table 4. Minimal Detectable Differences in Plasma Antioxidant Levels**

Antioxidant <sup>1</sup> (µg/ml)	Mean	SD	Minimal Detectable Difference	
			Absolute (Relative %)	
			80%	90%
Lutein/Zeaxanthin	0.21	0.084	0.043 (20%)	0.050 (24%)
β-Cryptoxanthin	0.08	0.048	0.025 (31%)	0.029 (36%)
Lycopene	0.30	0.141	0.073 (25%)	0.084 (28%)
α-Carotene	0.07	0.04	0.021 (30%)	0.024 (34%)
β-Carotene	0.33	0.031	0.016 (5%)	0.019 (6%)
α-Tocopherol	22.56	8.46	4.37 (19%)	5.05 (22%)

<sup>1</sup> Data are from Inserra *et al.*, 1999 (n=46)

In summary, we will have at least 90% power for detecting relative differences of 7-20% for primary and secondary biomarkers and 6-36% for plasma antioxidants.

#### 10.5 Statistical Analysis

For each patient in the trial, we will collect four types of data: (1) baseline personal characteristics and prognostic factors (tumor site and stage); (2) treatment; (3) SEBs; and (4) treatment-related toxicity or side effects. Descriptive statistics (means, standard deviations, percentages, etc.) for patient characteristics (age, race, and primary tumor site) and the outcome measures will be presented for each treatment group. Tables, graphs, and plots will be used to illustrate the data when appropriate. Graphical data description will serve two main purposes: (1) data exploration will enable us to understand the distribution of our variables particularly in the early stage of analysis; and (2) presentation of findings in a formal way.

**Specific Aim 1** will test the working hypothesis that F&V extracts can modulate SEBs in patients with previous HNSCC. We will quantify changes in SEBs associated with F&V treatment compared to naturally occurring changes, as estimated from the patients receiving the placebo. The primary outcome will be p27 immunohistochemistry staining. The secondary outcomes will be Ki-67 labeling index, DNA damage, and immune function. The primary analysis will focus on

testing post-treatment differences in these measures between the groups after adjustment for pretreatment levels. Analyses will also be done to assess changes over time within each treatment group. All tests will be two-sided;  $p \leq 0.05$  will be considered statistically significant.

Group Differences: Analysis of covariance will be used to assess group differences in our primary and secondary outcome measures after adjusting for pretreatment values and patient characteristics such as smoking status, alcohol use, primary tumor stage, age, sex, and race. Adjustments will be made to ensure the analyses match the design, to correct for chance imbalances in important prognostic factors, to improve the precision of the group comparisons by accounting for that part of the variance due to the variability in the patient characteristics, and to improve the acceptability of the results and to limit criticism of final publications (that any difference, or lack thereof, was due to chance imbalances in pretreatment values). Regression diagnostics, residual plots, and exploratory analyses will be done to find appropriate transformations for the variables in these analyses. Order of priority in choosing a transformation will be to satisfy the 1) linearity assumption, 2) homogeneity of variances assumption, and 3)

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normality assumption. Logistic regression will be used to assess differences in any dichotomous outcomes (e.g., toxicity - yes vs. no, biomarker increased - yes vs. no) between groups after adjustment for covariates.

Changes over Time: For each of these markers, the analyses will focus on comparing (within individuals) the pretreatment values to the values at 12 weeks post-treatment. Thus, the statistical evaluation will consider the mean difference between baseline and post-treatment, and test whether this difference is significantly different from zero. All SEB outcomes will be continuous. Analyses will be based on the paired *t*-test or, if the data do not conform to a Gaussian distribution after logarithmic or other appropriate transformations, the Wilcoxon signed rank test will be used.

Estimation and Confidence Limits: Although significance tests give one measure of the strength of evidence for treatment differences, they do not provide detail on the actual magnitude of the treatment difference. Thus, hypothesis tests will be followed by statistical estimation methods such as confidence limits (intervals). For example, we will present 95% confidence intervals for mean group differences to demonstrate the possible effect of F&V on SEBs compared with placebo. We will also present the 95% confidence limits for SEB means. For skewed random variables, the median will be presented as a descriptive measure of central tendency, and confidence intervals will be calculated using bootstrap methods.

**Specific Aim 2** will evaluate whether augmentation of p27 by F&V may be influenced by other factors, such as the characteristics of the original tumor (i.e., sites and stage), continued tobacco/alcohol use, and depression. This exploratory aim will be accomplished by including cross-product terms in the ANCOVA model. Interaction terms involving patient characteristics with treatment arm will also be included in other exploratory analyses to assess whether or not the effect of treatment on the other intermediate biomarkers is modified by other measurable factors.

**Specific Aim 3** will test the hypothesis that F&V can increase plasma antioxidant levels which may contribute to biomarker changes. Analysis of covariance, as described above, will also be used to assess the effect of F&V on antioxidant levels. Baseline levels of important antioxidants will be included as covariates in the analysis. Correlational analyses will be done to assess the relationships between the plasma antioxidants, the SEBs, and between the antioxidants and SEBs at each time. Simple associations between variables will be examined using bivariate plots, and spline fitting algorithms. Spearman and Pearson correlations will be calculated to quantitate the strength of the monotonic and linear relationships between continuous measures.

Additional Exploratory Analyses: Principal components analysis will be used to reduce the dimensionality of the biomarker and antioxidant measures and to reveal patterns in these outcomes. Multivariate analysis of covariance will be performed to assess group differences in meaningful contrasts of the multiple outcome measures.

Secondary Endpoints: The purpose of the exploratory tests on the secondary end points is to generate working hypothesis for future investigations, and, as such, each of these tests will be done using a 5% level of significance.

Toxicity and Adverse Events: Maximum toxicities and adverse events reported over the course of treatment will be assessed at the end of the study. Chi-square tests (or Fisher exact tests when numbers are small, as will likely be the case) will be used to assess unadjusted treatment differences in these outcomes. Exact logistic regression will be used to assess treatment differences in selected toxicities (as the number of events warrant) after adjustment for differences in other covariates.

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SEBs in Predicting Clinical Outcome: Although it is well-established that the SEBs under evaluation are related to the development and/or progression of HNSCC, the response to F&V measured by SEBs may or may not predict clinical outcome. To demonstrate the application of SEBs to predicting clinical outcome, it is necessary to conduct large Phase III studies using both SEBs and tumor recurrence as study endpoints.

**11.0 Sample Collection and Laboratory Procedures** (See Appendix XVII for site collection & shipping or oral biopsy and biomarkers)

**11.1 Sample Preparation and Transportation**

At baseline, following a complete head and neck examination (to include visualization of the oral cavity, oropharynx, hypopharynx, and larynx) by an otolaryngologist or a radiation oncologist, biopsies will be taken from the left side of the floor of the mouth. This area has been chosen for its accessibility. At 12 weeks, a biopsy will be obtained from the right side of the floor of the mouth. Biopsies will be obtained under local anesthesia using 1% lidocaine with epinephrine (approximately 0.2 ml). A single biopsy measuring approximately 1 cm x 2 mm will be obtained and divided into four pieces. The biopsy site will be inspected for hemostasis by the physician and treated accordingly; bleeding should be minimal. Closure of the biopsy site mucosa is at the physician's discretion. Biopsies will be clearly labeled with appropriate study identifiers, the biopsy site, and the type of assay. They will be teased out of the forceps onto bibulous paper so that they are not twisted or curled. One piece of the biopsy will be immediately fixed in 10% buffered formalin. The other three pieces of the biopsy will be frozen in dry ice immediately and transported to Dr. Hu's laboratory and stored in liquid nitrogen.

A 20 ml sample of whole blood (three purple-top, 8 mL tubes and one yellow top, 5 mL tube; Vacutainer Systems; Becton Dickinson) will be obtained by venipuncture during the clinical examination and transported immediately to the laboratory for processing and storage. The blood samples will be processed within two hours of phlebotomy. For an analysis of the vitamins A and E, and carotenoid profiles, 4 mL of blood will be collected in tubes containing clot activator and further prepared on ice under subdued light. Within 30 min, the tubes will be centrifuged. Serum samples for analysis of vitamins A and E and carotenoids will be stored at -70° C until analysis. For analysis of vitamin C, immediately after collection, 0.5 mL blood will be added to 2.0 mL metaphosphoric acid (50 g/L) under continuous vortexing. This mixture will be placed on dry ice, stored at -70° C, and analyzed within 5 days. The remaining blood (about 15 mL) will be used for lymphocyte isolation. Lymphocytes will be cryopreserved and stored at -140° C until future assays.

Sample Packaging, Tracking, and Responsibilities: All the study samples will be packaged and transported by carrier service immediately after samples were taken following the government regulation 42 CFR Part 72 – “Interstate Shipment of Etiologic Agents”, which describes the requirements for the proper packaging and shipping of infectious substances and other biomedical material. All the samples will be packaged and delivered in such a way that the contents will not leak and will arrive in good condition. Each blood tube will be placed in a plastic, screw-top container (with cotton ball in bottom), secure all samples together with rubberband, placed in tray in insulated cooler. The blood will be transported in a small insulated cooler at temperature between 50-70 degrees F with the biohazard label on the front to Dr. Hu's laboratory for processing. A single biopsy measuring approximately 1 cm and 2 mm thick will be obtained and divided into four pieces. Biopsies will be clearly labeled with appropriate study identifiers and the biopsy site. One piece of the biopsy will be immediately fixed in 10% buffered formalin and transported with the blood samples at temperature between 50-70 degrees F using the cooler. The other three pieces of the biopsy will be frozen in dry ice immediately and transported in dry

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ice packed in a with the biohazard label on the front to Dr. Hu's laboratory and stored in liquid nitrogen. The research nurse at each site will call Dr. Hu's lab Tel: 336-713-7645 or 336-713-7649 to let them know the samples will be coming. The shipping address is as follows:

Laura Gilliam, RN, BSN/Jennifer J. Hu  
Department of Cancer Biology, NRC-446  
Wake Forest University Health Sciences  
Medical Center Blvd.  
Winston-Salem, NC 27157  
Telephone: (336) 713-7645 or (336) 713-7649

Dr. Hu's laboratory personnel will process the biological samples Monday through Friday during normal working hours. Do not ship to arrive on a Saturday, Sunday or holiday. The research nurse needs to call if late samples will be delivered. The laboratory will log the samples in the log sheet located in the laboratory and process the samples immediately. Microsoft Access database will be use to track all the samples.

### 11.2 Plasma Antioxidant Levels

Dr. Miller's laboratory will perform the assay. The lipid soluble vitamins, retinol, tocopherols, carotenes, lycopene and xanthophylls will be analyzed according to a modification of the methods by Hess *et al.* (1991) and Aebisher *et al.* (1999). Briefly, in subdued yellow/orange lighting, 200  $\mu$ L of plasma or standard solution and internal standard will be deproteinated with BHT (butylated hydroxy toluene) ethanol and buffer and extracted twice with hexane under argon. The combined upper organic layers will be dried under nitrogen and reconstituted with 200  $\mu$ L THF (tetrahydrofuran)/BHT ethanol. The HPLC system consists of a Waters 600 pump, 717+ automatic injector, 474 fluorescence detector and Millennium software. A second detector, a programmable multi-wavelength Spectra-system UV/Vis 2000, will also be used. Two detectors are required to monitor all the necessary wavelengths to quantitate the analytes at their absorbance and excitation/emission maxima to insure sensitivity. Separation of the analytes will take place on a Beckman ODS 5 $\mu$ m, 250 x 4.6 mm column. The isocratic mobile phase consists of 680 ml acetonitrile, 220 ml tetrahydrofuran, 68 ml methanol and 28 ml of a 1% ammonium acetate solution. The flow rate is 1.2 mL/min. Samples will be maintained at 8°C in the automatic injector. The analyte limits of detection were determined by Hess *et al.* (1991): alpha-carotene 10  $\mu$ g/L, beta-carotene 10  $\mu$ g/L, beta-cryptoxanthin 10  $\mu$ g/L, lycopene 5  $\mu$ g/L, retinol, 20  $\mu$ g/L, alpha-tocopherol 0.05 mg/L, gama-tocopherol 0.05 mg/L. Final determination of each working stock solution concentration will be determined spectrophotometrically using the appropriate extinction coefficient. A seven point standard curve along with low and high controls will be run every day with patient samples. Assays where the controls vary > 10% of expected values will be repeated.

### 11.3. Histopathologic Evaluation and Immunohistochemical Assays (IHC)

Dr. Willingham's laboratory will perform histopathologic evaluation, immunohisto-chemical assays, and image analysis. Standard microscopic examination of all tissue biopsies will be done using established cytologic and architectural pathologic criteria to differentiate reactive from dysplastic changes. Formalin-fixed tissue will be paraffin-embedded and 4  $\mu$  sections will be cut and put onto plus slides (Baxter, Charlotte, NC). These sections will be air dried, deparaffinized with xylene, hydrated, and antigen retrieved. Then the slides will be 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 will be counterstained with Gill's

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hematoxylin and analyzed for percent of cells stained and intensity of stain. In each study, a negative control will also be run, where the primary treatment contains no primary antibody.

#### 11.4 Computer-Assisted Image Analysis

Computer-assisted image analysis will be used to quantitate the intensity of nuclear label and the percentage of the squamous epithelial cells showing peroxidase label. The squamous epithelial layer present in the oral biopsies will be evaluated for both intensity of nuclear label and percentage of the squamous epithelial cells showing peroxidase label as follows: brightfield color digital images using identical illumination methods between samples will be captured using a 10X, 20X, and 40X objective with a SPOT-cooled CCD camera. These digital images will then be imported into Adobe Photoshop software on a Dell GXPro workstation. Each image will be selected to encompass only the thickness of the epithelial layer. Within these selected areas, the Photoshop "magic wand" tool will be used to select a threshold area that encompasses all nuclei visible with hematoxylin (total nuclear area) and then nuclei that contain the brown color set characteristic of peroxidase DAB reaction product (labeled nuclear area).

The number of pixels encompassed in each category in the histogram menu will then be recorded as well as the mean density of the labeled histogram. The same evaluation will be made along the entire length of the available squamous layer in each biopsy, usually requiring 4-5 fields at 40X and a minimum of 50,000  $\mu\text{m}^2$  of nuclear area per case. A similar evaluation will be made for each negative control sample. These values can then be subtracted from the antibody sample to generate specific labeling values. From these numbers, a product index will be generated that combines both the intensity and the percentage of nuclei labeled as well as the individual numbers in each intensity and percentage data set. Using these methods, several hundred nuclei can be evaluated quantitatively for each biopsy sample.

#### 11.5 DNA Damage - Comet Assay

Dr. Hu's laboratory will perform the single-cell electrophoresis assay (Comet assay) for the quantitation of DNA damage and repair in PMLs in response to 3 Gy of  $\gamma$ -ray or 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  to generate oxidative damages and strand breaks (Smith *et al.*, 2001). For alkaline lysis, cells will be suspended in low melting point agarose, deposited onto the Comet slide surface, and gently lysed with a high-salt and detergent lysis solution at pH 10 for alkaline lysis. Lysis will be followed by DNA unwinding in which the cells will be treated in a highly alkaline solution, forming apurinic/apyrimidinic sites. For the neutral version of the assay, slides will be submerged for 2.5 hours in 1% sarkosyl, 2 M NaCl, and 30 mM EDTA. After lysis, slides will be rinsed overnight in Tris-borate-EDTA buffer (Olive *et al.*, 1990). After unwinding, all the slides will be submerged in a standard horizontal gel electrophoresis apparatus. Following electrophoresis, the slides will be removed, fixed in ethanol, and dried. Staining will be accomplished using SYBR<sup>®</sup> Green, which provides high sensitivity. For each study subject, we will collect data on basal DNA damage (without any treatment) and IR- or  $\text{H}_2\text{O}_2$ -induced DNA damage. For quantitation, the comet tail moment of 100 cells will be analyzed using the image analysis package, LAI Automated Comet Assay Analysis System (LACAAS; Loats Associates, Inc., Westminster, MD).

#### 11.6 T-cell Response to IL-2

Dr. Hu's laboratory will perform the T-cell proliferation assay in response to IL-2 using the method described previously (Pandit *et al.*, 2000). PMLs will be seeded into microtiter wells containing 100 U/mL, 10 U/mL, 1 U/mL, or 0 U/mL of IL-2. The cells will be incubated for 5 days at 37 C. Proliferation will be measured using a tetrazolium-based colorimetric assay (Cell Titer96 AQueous Cell Proliferation Assay, Cat#5440, Promega Corp, Madison, Wisconsin). The CellTiter 96AQueous

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assay is a colorimetric method for determining the number of viable cells in proliferation, cytotoxicity, or chemosensitivity assays. The CellTiter 96 AQueous Assay is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS, and an electron-coupling reagent (phenazine methosulfate) PMS. MTS is bioreduced by cells into a formazan that is soluble in tissue culture medium. The conversion of MTS into the aqueous soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

### 11.7 Characterization of Chemopreventive Agents

Dr. Thomas laboratory will perform the HPLC assays to characterize potential chemopreventive agents present in Juice Plus™. Most of the earlier studies demonstrated the presence of major carotenoids and antioxidants important for general health (Wise *et al.*, 1996; Inserra *et al.*, 1999; Smith *et al.*, 2000). However, it is important to characterize F&V-specific agents with additional chemopreventive properties. We will test two groups of compounds in the F&V extracts and blood: (1) glucosinolates, isothiocyanates (ITC) and dithiocarbamates (DTC): *Cruciferae* and 15 other plant families contain high concentration of glucosinolates, which are the precursors of ITC; (2) flavonoids (i.e., quercetin, hesperetin, naringenin, anthocyanins, flavonol, and proanthocyanidins): present in apples, cranberries, kale, cabbage, and other plants.

ITC/DTC Determination: ITC and DTC react quantitatively with 1,2-benzenedithiol to produce 1,3-benzodithiole-2-thione and this cyclocondensation reaction can be used to determine DTC/ITC levels in plant material, urine, blood and tissues (Zhang *et al.*, 1996; Ye *et al.*, 2002). The 2.0-ml reaction mixtures will contain 500 µl of 100 mmol/l potassium phosphate buffer, pH 8.5, 1.0 ml of 20 mmol/l 1,2-benzenedithiol in acetonitrile and 500 µl of the test sample. The mixture will be incubated for 2 h at 65C to ensure that all ITC and DTC are converted quantitatively to 1,3-benzodithiole-2-thione. After cooling to room temperature and low-speed centrifugation to sediment any precipitates, a 200-µl aliquot of the supernatant fluid will be injected onto the reverse phase HPLC column (Partisil 10 ODS-2, Whatman, Clifton, NJ), and eluted with 80% methanol/20% water at a rate of 2 ml/min. The 1,3-benzodithiole-2-thione will be eluted at about 5 min, and the peak will be detected and integrated by Photodiode array detector (Waters Model 996) at 365 nm. The instrument will be calibrated with pure 1,3-benzodithiole-2-thione. In the previous publication (Ye *et al.*, 2002), the lower limit of detection of the integrated area of the cyclocondensation product peak was 5 pmol.

Flavonoids Determination: In a combined database from Finland and Netherlands, flavonoids included were 4 flavonols (kaempferol, quercetin, myricetin, and isorhamnetin), 2 flavones (apigenin and luteolin), and 3 flavanones (hesperetin, naringenin, and eriodictyol). (Knekt *et al.*, 2002). Quercetin is mainly provided by apples and onions and kaempferol by white cabbage. Hesperetin and naringenin are derived from citrus fruit and myricetin from berries. The intakes of apigenin, luteolin, isorhamnetin and eriodictyol were very low (Knekt *et al.*, 2002). In addition, a recent study showed that the plasma levels of these three flavonoids were significantly increased in human subjects following diets high in F&V (Erlund *et al.*, 2002). Therefore, we will focus on three flavonoids, quercetin, hesperetin, naringenin for this study. A HPLC method with in-line connected diode-array (DAD) and electro-array (EC) detection will be used to identify and quantify 17 flavonoids (Mattila *et al.*, 2000). Catechins will be extracted from the samples using ethyl acetate, and quantification of these compounds will be performed with the EC detector. Other flavonoids will be quantified with DAD after acid hydrolysis.

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The ITC/DTC and flavonoids profiles will be used to support our hypothesis that many potential chemopreventive agents in addition to antioxidants and carotenoids found in F&V contribute to their chemo-protective properties. More importantly, they can also be used to determine batch-to-batch variations in the future as part of the quality control effort.

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