CLINICAL PROTOCOL

Study Title: Randomized Clinical Trial of Lung Cancer Chemoprevention with Sulforaphane in Former Smokers

NCT03232138

September 23, 2019 Version 11.

PRINCIPAL INVESTIGATOR	CO-PRINCIPAL INVESTIGATOR
Jian-Min Yuan, M.D., Ph.D. Professor Division of Cancer Control and Population Sciences University of Pittsburgh Cancer Institute 5150 Centre Avenue UPMC Cancer Pavilion, Suite 4C Pittsburgh, PA 15232 (412) 864-7889 yuanj@upmc.edu	**David O. Wilson, M.D.** Associate Professor Division of Pulmonary, Allergy and Critical Care Medicine, School of Medicine University of Pittsburgh Cancer Institute 5150 Centre Avenue UPMC Cancer Pavilion, Suite 4C Pittsburgh, PA 15232 (412) 687-3355 wilsondo@upmc.edu

CO-INVESTIGATORS

Sanja Dacic, M.D., Ph.D. Professor Department of Pathology School of Medicine UPMC-PUH, Room C608 200 Lothrop Street Pittsburgh, PA15213 (412) 647-8694 dacics@upmc.edu	**James Herman, M.D.** Professor Division of Hematology/Oncology University of Pittsburgh Cancer Institute 5117 Centre Avenue 2.18d Hillman Cancer Research Pavilion Pittsburgh, PA 15232 (412) 623-7769 hermanj3@upmc.edu
Thomas W. Kensler, Ph.D. Professor Department of Pharmacology and Chemical Biology, School of Medicine E1352 Thomas E. Starzl BST 200 Lothrop Street Pittsburgh, PA15213 (412) 648-1942 tkensler@pitt.edu	**Daniel Normolle, Ph.D.** Associate Professor Department of Biostatistics UPMC Hillman Cancer Center Biostatistics Facility 201 North Craig Street Pittsburgh, PA 15213 (412) 383-1591 dpn7@pitt.edu
Shivendra Singh, Ph.D. Professor Department of Pharmacology and Chemical Biology, School of Medicine University of Pittsburgh Cancer Institute 5117 Centre Avenue 2.32A Hillman Cancer Research Pavilion Pittsburgh, PA 15232 (412) 623-3262	**Avrum Spira, M.D., M.Sc.** Department of Medicine, Pathology and Bioinformatics Boston University School of Medicine 72 East Concord Street, E-631 Boston, MA 02118-2308 (617) 414-6980 aspira@bu.edu

singhs@upmc.edu	

TABLE OF CONTENTS **2**

1. OBJECTIVES

1.1 Primary Aims

1.1.1 To recruit 72 former smokers at high risk of developing lung cancer who will be randomly assigned to either receiving Avmacol® or placebo tablets for 12 months. Bronchoscopy-guided bronchial biopsy and brushing, bronchoalveolar lavage, nasal brushing, blood and urine samples will be collected from each subject at pre- and post-intervention time points.

1.1.2. To determine if daily oral dose of 120 micromole sulforaphane (SF) for 12 months can modulate the changes of bronchial dysplasia index, cell proliferation marker Ki-67, and apoptosis markers caspase-3 and TUNEL in bronchial biopsies in former smokers at high risk for lung cancer.

1.2 Secondary Aims

1.2.1 To explore if daily oral dose of 120 micromole SF for 12 months can modulate the changes of the lung cancer-related gene expression markers in bronchial epithelia in former smokers at high risk for lung cancer.

1.2.2 To explore if daily oral dose of 120 micromole SF for 12 months can modulate the changes of bronchial premalignant lesions-related gene expression markers in former smokers at high risk for lung cancer.

1.2.3. To explore if daily oral dose of 120 micromole SF for 12 months can modulate the similar changes of the gene expression markers in nasal epithelia as in the bronchial epithelia identified in Aims 1.2.1 and 1.2.2.

1.1.4 To determine the safety and toxicity of daily oral dose of 120 micromole SF for 12 months in former smokers at high risk for lung cancer.

2. BACKGROUND

2.1 Rationale for sulforaphane as a chemopreventive agent for lung cancer

Lung cancer is leading cause of lung cancer death in both men and women worldwide (1.5 million deaths) and in the US (158,000 deaths) (2,3). Cigarette smoking causes 90% of lung cancer (4). Smoking cessation is an obvious and practical strategy for lung cancer prevention for smokers. The smoking cessation programs and antismoking campaign have resulted in significant reduction in prevalence of cigarette smoking in the US, from 60% in late 1960s to 17% in 2015; currently there are more former smokers than current smokers (5). However, former smokers remain at high risk of lung cancer even after they quit smoking for many years (6). In fact, the majority of new lung cancer cases occur among former smokers (7,8). Unfortunately the preventive intervention for lung cancer targeting former smokers is lacking.

Epidemiological studies have shown that intake of isothiocynantes (ITCs), natural products formed from glucosinolates found in certain cruciferous vegetables such as broccoli and its seeds, are associated with a reduced risk of lung cancer. Using a validated biomarker approach (9,10), we, for the first time, demonstrated that high urinary total ITCs, including both sulforaphane (SF) and phenethyl isothiocyanate (PEITC), were associated significantly with a 35% reduced risk of lung cancer (11). These novel findings stimulated many epidemiological and experimental studies on the potential lung cancer protection of dietary ITCs and cruciferous vegetables in many populations. A meta-analysis including 14 studies and more than 8,000 lung cancer cases reported a statistically significant 20% reduced risk of lung cancer for subjects with high intake of cruciferous vegetables (12). These data implicate a protective role of ITCs or cruciferous vegetables as a whole against the development of lung cancer in humans. However, epidemiological studies are inherent with measurement errors and potential confounding, thus the observed association between ITCs and lung cancer risk may not be causal. Furthermore, results from observational studies usually do not provide a biological underpinning.

Preclinical data in rodent models is quite persuasive for prevention of lung cancer with ITCs. In an experiment by Hecht *et al*. (13), 160 rats were assigned to 4 groups: 1) 60 rats treated by tobacco-specific lung carcinogen NNK (2 ppm) only, 2) 60 rats of NNK (2 ppm) plus PEITC (3 micromole/g diet), 3) 20 rats of PEITC (3 micromole/g diet) only, and 4) 20 rats untreated as controls. PEITC was added to diet at a non-toxic concentration for the entire study period (112 weeks) whereas NNK was added to the drinking water one week after the initial administration of PEITC. At the end of the study (after 112 weeks of PEITC and 111 weeks of NNK treatment), 70% of the NNK-treated only rats developed lung adenoma and adenocarcinoma while only 5% of rats treated with PEITC and NNK developed lung tumors, similar to those in the PEITC-treated only or untreated control rats. These results showed that PEITC, treated concurrently with NNK, a setting analogous to the situation of current smokers who are continuously exposed to tobacco carcinogens including NNK, completely inhibited carcinogen-induced lung tumors in rats.

In another experiment by Chung and colleagues (14) with a different study design, mice were treated with a mixture of tobacco carcinogens: 3 micromole benzo(a)pyrene [B(a)P] and 3 micromole NNK, given gavage once a week for 8 weeks first, stopped carcinogen treatment for 12 weeks, and then fed a diet containing different concentrations of sulforaphane (SF) (1.5 or 3.0 micromole/g diet) or phenethy isothiocyanate (PEITC) (1.5 or 3.0 μmole/g diet), for 22 more weeks. At the end of the study, the incidence of lung adenocarcinoma was reduced by a statistically significant 52% in mice treated with carcinogens plus SF, and by 55% in mice treated with carcinogens plus PEITC, compared with mice treated with carcinogens only. The tumor multiplicity was reduced by 70% in mice treated with carcinogens plus SF and by 60% in mice treated with carcinogens plus PEITC. These results show that both dietary SF and PEITC can inhibit the development of lung tumors induced by two major tobacco lung carcinogens – B(a)P and NNK. More importantly, the

chemopreventive agents SF and PEITC were given 12 weeks after the carcinogen treatment was stopped, a setting analogous to a situation of exposure for former smokers.

Besides the inhibition of tumor incidence and multiplicity, both SF and PEITC can inhibit cellular proliferation markers such as Ki-67 and induction of apoptosis (15), hallmarks of tumorigenesis. In the experiment by Chung and colleagues (14), dietary intake of SF (1.5 micromole/g diet) reduced number of lung cells expressing proliferative cell nuclear antigen, an alternative to Ki-67, by 61% (from 44.4 ± 1.9 to 17.3 ± 11.7) (14). In a double-blinded randomized clinical trial, women with abnormal mammograms and scheduled for breast biopsy who consumed SF precursor GR supplement (estimated daily urinary excretion of 9.7 μmole total metabolites of SF) for 2-8 weeks showed significantly reduced Ki-67 labeling indices (LI) in breast benign tissue (16). Dietary SF and PEITC also induced markers of apoptosis in lung tissue of mice after treated with tobacco carcinogens. Compared with control animals, mice given SF (1.5 micromole/g diet) showed a statistically significant 6-fold increase (from 4.7±1.9 to 29.3±4.7%) in activated caspase-3, and a 2-fold increase (from 12.7±3.0 to 29.4±5.8%) in TUNEL (terminal transferase dUTP nick end 7labelling) of lung cells (14).

We recently completed two randomized, double-blinded, placebo-controlled clinical trials. The first trial was led by Dr. Kensler to evaluate whether intake of SF enhances the detoxification metabolism of environmental carcinogens and toxicants such as benzene and acrolein in 283 subjects (89% were non-smokers) who were exposed to substantial levels of airborne pollutants in China. Intake of a broccoli sprout-derived beverage providing daily doses of 600 micromole GR and 40 micromole SF for 12 weeks significantly increased the urinary excretion of the detoxification mercapturic acids formed from benzene, an environmental carcinogen present in both tobacco smoke and polluted air, by 61% ($P<0.001$) and acrolein by 23% ($P=0.01$) (17). The median levels of the 24-hour urinary excretion of SF metabolites with this dosing regimen were 54-62 micromole across the intervention period (17). The second trial was led by Dr. Yuan to evaluate if intake of PEITC inhibits the metabolic activation of tobacco-specific carcinogen NNK and enhances the detoxification metabolism of tobacco-nonspecific carcinogens and toxicants such as benzene and acrolein. Overall, intake of PEITC 40 mg/day for 5 days significantly inhibited NNK metabolic activation by 8% ($P = 0.023$), and increased urinary detoxification mercapturic acids formed from benzene by 25% ($P = 0.002$) and acrolein by 15% ($P = 0.005$), in 82 current smokers in the US. The mean level of the 24-hour urinary excretion of PEITC-NAC was approximately 80 micromole. These very similar results of the two trials on the detoxification of environmental carcinogens and toxicants suggest that both SF and PEITC have a very similar biological mechanism and share the strongly overlapping chemopreventive properties, as shown in the animal experiment (14).

These two randomized phase 2 clinical trials provided crucial data on the safe and effective dose and duration of treatment. The unbiased results of these trials elucidate the modulating effect of these ITCs on the metabolisms of tobacco-specific (NNK) and nonspecific carcinogens (benzene) and toxicants (acrolein) through specific

biological pathways. These encouraging results are very important for further development of these ITCs as primary chemopreventive agents against the development of lung cancer. However, these results are systemic and related to actions at the upstream on the lung carcinogenesis pathway. Before we can launch primary chemoprevention studies against the development of lung cancer as an end point, we need to demonstrate if ITCs such as SF can modulate the changes of downstream biomarkers on the lung carcinogenesis pathway such as bronchial dysplasia, cell proliferation and apoptosis, and gene expression in bronchial biopsies that have been shown to be directly linked to the development of lung cancer (18).

We propose to further evaluate the chemopreventive effect of SF against lung carcinogenesis through a randomized, double-blinded, placebo-controlled phase 2 clinical trial, a proof-of-principal study, with a longer treatment period (12 months) for former smokers who are at high risk for lung cancer. We hypothesize that intake of SF will improve pathological features of bronchial dysplasia, decrease cell proliferation and increase apoptosis in endobronchial biopsies, and improve the gene expression profile of lung cancer-related genes in endobronchial epithelial cells, all of which have been shown to predict lung cancer development and diagnosis (18,19). Successful outcome of this systemic analysis is crucial to further develop SF as a chemopreventive agent for the primary prevention of lung cancer. Our ultimate goal is to validate the efficacy of this readily available, widely accessible, inexpensive, natural compound as a primary chemopreventive agent against the development of lung cancer in humans.

Findings from epidemiological and animal experimental studies as well as from short-term randomized phase 2 clinical trials have shown cancer chemoprotective properties of sulforaphane. Although these results are encouraging, they are not sufficient and justifiable for launching large randomized phase 3 clinical trials to evaluate the efficacy of SF on reduction of lung cancer incidence and mortality as the primary endpoints, which require large amounts of resources and many years of intervention and many more years of post-intervention follow-up. We, the research community, have learned hard lessons from failed large placebo-controlled phase 3 chemoprevention trials when their premises were primarily based on the observed inverse association from epidemiological studies, but not by strong biological mechanisms supported by pre-clinical studies in animals and early phase randomized clinical trials in humans. These previously failed trials included CARET (beta-carotene and retinol) and ATBC (beta-carotene and alpha-tocopherol) (20,21)}. Therefore, direct evidence of a protective effect of SF on bronchial cellular and molecular biomarkers proposed here is crucial for further development of SF as a primary chemoprevention agent against the development of lung cancer in humans. The proposed study, if it proves our hypotheses to be true, will have significant public health implications on reduction of lung cancer incidence and mortality in former smokers.

2.2 Prior chemoprevention studies

There have been a limited number of phase 2 chemoprevention studies in lung cancer. Among them were the Iloprost and Celecoxib trials. We participated in the multicenter double-blind, randomized, placebo-controlled phase 2 trial of oral iloprost, a prostacyclin analogue, in current and former smokers with sputum cytological atypia or endobronchial dysplasia (22). Bronchoscopy was performed at study entry and after completion of the six-months treatment. Within each subject, we calculated the average histological score of all biopsies, the worst biopsy score, and the dysplasia index. Compared with former smokers in the placebo group, former smokers treated with oral iloprost exhibited a significantly greater improvement in average score (0.41 units better, P = 0.010), in the worst biopsy score (1.10 units better, P = 0.002), and in the dysplasia index (-12.5%, 95% CI -21.0% to -3.9%, P = 0.006). The 6-month treatment with iloprost did not show any improvement of histological scores in current smokers (22). Endobronchial dysplasia is a presumed precursor of non-small cell lung cancer (NSCLC). In a follow-up study of a high-risk cohort of subjects with endoscopic biopsies, persistence of bronchial dysplasia was associated significantly with a 7-fold increased risk of developing NSCLC (adjusted hazard ratio = 7.8, 95% CI 1.6-39.4). Furthermore, elevated Ki-67 LI at baseline endobronchial biopsies were associated significantly with increased bronchial dysplasia score, which predates the development of lung cancer (18).

Another similar phase 2 clinical trial was the celecoxib trial conducted by Mao and colleagues (23). The study enrolled former smokers (age ≥45 years, ≥30 pack-years of smoking, ≥1 year of sustained abstinence from smoking) to evaluate the impact of COX-2 selective inhibitor celecoxib on cellular and molecular events associated with lung cancer pathogenesis. The primary endpoint was bronchial Ki-67 LI after 6 month treatment. Celecoxib significantly reduced Ki-67 LI by an average of 34% (P = 0.04). Furthermore the decrease in Ki-67 LI correlated with a reduction and/or resolution of lung nodules on CT (P = 0.008).

Although both iloprost and celecoxib have shown effective on the reduced regression of bronchial dysplasia in former smokers (22-24), both are not available for further development as potential chemopreventive agents due to their toxicity (celecoxib) or lack of readily deliverable form such as tablets (iloprost). Sulforaphane is a promising chemopreventive agent, a natural compound with minimal side effects, readily available, widely accessible, and inexpensive for mass production. Smaller trials with intermediate endpoints in high-risk subjects are needed to provide crucial data for further development of SF as a primary chemoprevention agent against the development of lung cancer in humans. The proposed study, if it proves our hypotheses to be true, will have significant public health implications on reduction of lung cancer incidence and mortality in former smokers.

3. PATIENT SELECTION

Seventy-two volunteers will be recruited for this phase II clinical trial.

3.1 Eligibility Criteria

1) Man or woman 55-74 years of age.
2) Patients with normal endobronchial biopsy findings or pre-cancerous lesions at baseline will be eligible for the study. Pre-cancerous lesions include (a) reserve cell hyperplasia, (b) squamous metaplasia, (c) mild dysplasia, (d) moderate dysplasia, and (e) severe dysplasia.
2) A former smoker who has a history of smoking with ≥30 pack-years, quits smoking within the past 10 years, and has more than 1 year sustained abstinence from smoking.
3) Female subjects must be of non-child bearing potential or must have a negative urine pregnancy test at screening (within 72 hours of first dose of study medication) if of childbearing potential.
4) Male and female subjects of childbearing potential must be willing to use adequate barrier methods of contraception from the time starting with the screening visit through 30 days after the last dose of study therapy.
5) Abstinence is acceptable if this is the established and preferred contraception for the subject.
6) Generally healthy with liver enzyme and blood count values within the ranges shown below on the blood sample drawn at the baseline screening visit. Specifically:

White blood cells	≥ 3,000/mL
Total bilirubin	≤ 1.5 x ULN (upper limits of normal)
AST (SGOT)/ALT (SGPT)	≤ 2.5 x ULN
BUN and serum creatinine	≤ 1.5 x ULN

7) The presence of airflow obstruction on spirometry (GOLD 1-4 & FEV1/FVC<0.7 &FEV1 <0.8) COPD; and/or any emphysema on CT scan.
8) Participants must have a Southwest Oncology Group (SWOG) performance status of 0-2 (Appendix 10).
9) Participants must be able and willing to undergo a bronchoscopy before and after treatment for 12 months.
10) Patients must be fully informed of the investigational nature of this study and must sign an informed consent in accordance within institutional and regulatory guidelines.

3.2 Exclusion Criteria

Patients are not eligible if any one of the following conditions exists:
1) Carcinoma in situ or invasive cancer on baseline endobronchial biopsy.
2) A malignancy except for adequately treated basal cell or squamous cell skin cancer or in situ cervical cancer.
3) Severe lung disease or inability to undergo two bronchoscopies.
4) Had pneumonia or acute bronchitis within the past 2 weeks prior to the date of enrollment (i.e., signing the consent form).
5) Had myocardial infarction (MI) or other severe heart diseases such as ventricular tachycardia, multifocal premature ventricular contractions or supraventricular tachycardias with a rapid ventricular response within the past 6 weeks prior to enrollment.
6) Hypoxemia (less than 90% saturation with supplemental oxygen).

7) Prior chemotherapy or thoracic radiation within the past 5 years.
8) Woman who is pregnant or plan to be pregnant in next 12 months, or is breast feeding or plan to begin breast feeding in next 12 months.
9) Life expectancy of < 12 months.
10) Have a history of irritable bowel disease such as Crohn's disease and ulcerative colitis.

3.3 Recruitment Methods

Patients for this trial will be recruited primarily from the participants of the Pittsburgh Lung Screening Study (PluSS) at the UPMC Hillman Cancer Center. This unique high-risk PluSS cohort enrolled approximately 3,600 current and former smokers in Pittsburgh area. The initial contact with the eligible subjects for the proposed study will be mailed a letter, followed up by a telephone call made by PluSS staff members who will briefly explain to the study subjects about the clinical trial and complete an eligibility screening form. Alternatively Any patients who are interested in participating in the study can telephone the clinical investigation office at 412-623-3317 Monday through Friday, 9:00 am to 5:00 pm (Eastern Time). The clinical research assistant will conduct a telephone interview with the potential study participant using the Eligibility Screening Form (Appendix 1). If all the requirements are met, the clinic research assistant will mail the following documents for subject to review before she or he has a scheduled clinical visit:

1) Locator Form (Appendix 2)
1) The Informed Consent Form (Appendix 3).
2) Baseline Medical History Questionnaire (Appendix 4).
3) Questionnaire for Cruciferous Vegetables (Appendix 5)

3.4 Consent Procedure

At the screening clinical visit (stage 1-visit 1), a clinical research nurse practitioner and/or the project manager will go over with the study subjects about the study, eligibility criteria, procedures, and potential risk and benefits in participating in the study in a setting that is quiet and unhurried. The project manager will go over the written informed consent form with the study subject and answer any questions the patient may have. The authorization describes in detail what information, including the HIPPA-protected information, will be collected and for what purposes it will be used. A copy of signed consent will be provided to subject.

3.5 Patient Payment

Subjects will receive no health benefit from participating in this research study. If SF is approved to be chemopreventive, subjects assigned to treatment arm may have some chemopreventive benefit from Avmacol®. Subjects will receive $500 for each research bronchoscopy at baseline and at the end of the treatment. In addition, we will pay participants $50 for each of 6 scheduled clinical visits from stage 1-visit 1 to statge 2-visit 6 to cover their costs of transportation and making effort to participate in the study. Parking for all clinical visits will be provided free of charge to the study

participants. At the end of the study treatment, the subject will receive $500 as bonus for the compliance and completion of the study (see Appendix 6. Participant Compsentation Form).

3.6 Registration Process

After being deemed eligible, participants will be assigned a patient number and be registered into the CTMA database.

4. STUDY AGENT INFORMATION

4.1 Broccoli seed preparations (BSP)

4.1.1 Summary

Broccoli seed preparations (BSP) have been studied extensively in multiple Phase I and Phase II clinical trials without Grade 2 or higher adverse effects. Broccoli seeds contain high concentrations of glucoraphanin (GR), the glucosinolate precursor of sulforaphane (SF), which is converted to SF by the release of intrinsic seed myrosinase during chewing or by thioglucosidaes in the microbiota in the human gut (25-27). The isothiocyanate SF (1-isothiocyanato-4*R*-(methylsulfinyl)butane) was originally isolated from commercial broccoli, and is one of the most potent naturally-occurring inducers of cytoprotective enzymes identified to date (28). SF activates the KEAP1-NRF2 signaling pathway, thereby increasing transcription of antioxidative and detoxication genes. For example, SF has been shown to induce cytoprotective enzymes in a variety of animal and human cell lines and tissues (29).

In our initial clinical trials, we investigated BSP in the form of re-hydrated, previously lyophilized broccoli sprout powders rich in either GR or SF produced by the Cullman Chemoprotection Center at Johns Hopkins under the supervision of Drs. Jed Fahey and Paul Talalay. Broccoli sprouts (*Brassica oleracea*) were grown from specially selected BroccoSprouts™ seeds to provide a consistent yield of GR or SF. In our initial trial, the GR-rich powder was considered as a food by the Johns Hopkins IRB. It is simply the freeze-dried powder derived from a hot water extract of broccoli sprouts. In 2010, the US FDA made a determination that beverages containing SF-rich broccoli sprout powders (but not GR-rich broccoli sprout powders) must be considered as drugs because the SF is formed ex vivo from the plant by the addition of myrosinase-containing daikon seeds during the preparation of the SF-rich powder. Thus, subsequent trials in Qidong, China (NCT01437501) as well as the pilot study at the University of Pittsburgh (NCT 02023931) have been conducted with an IND from the FDA (#112038) held by Thomas Kensler PhD (Co-I).

There are three main problems in achieving consistent delivery and bioavailability of active SF by means of lyophilized broccoli sprout extracts: (1) SF is only moderately storage- and heat-stable over time, especially in aqueous solution. (2) SF-rich broccoli sprout extract powders are extremely hygroscopic and the preparation of

capsules containing accurate doses by weight is challenging and very expensive. (3) Administration of GR as an oral precursor of SF results in highly variable conversions of GR to SF metabolites (dithiocarbamates) among individuals, ranging from 1 – 40%, but fairly consistent between individuals (17,27,30-32).

In this study in healthy former smokers, BSP will be provided in the form of the commercially available dietary supplement, Avmacol®. Although a number of dietary supplements containing GR have been available in the U.S. market for several years now, Avmacol® is the first commercial product to contain GR plus the fully active enzyme myrosinase, thus yielding a higher and much more consistent SF dose upon ingestion. Avmacol® tablets contain only GR-rich broccoli seed extract, freeze-dried broccoli sprouts for the myrosinase source, and the inert excipients required to form a tablet. They are manufactured by Nutramax Laboratories, Inc. (Edgewood, Maryland, USA). Avmacol® has been sold as a nutritional supplement in the United States since 2013, and is manufactured under GMP standards, which has a shelf life of two years at ambient temperature. Based on previous human studies with both dietary and topical broccoli sprout extracts, and related broccoli seed preparations, and the absence of severe or serious risks (no Grade II toxicities have been observed in > 500 patients) (see Table 3 in Clincal Protocol) , we do not anticipate serious adverse events to be associated with the administration of Avmacol® to study participants in this proposed trial.

4.1.2. Description of Origin of Sulforaphane, Purification, and Stabilization

Sulforaphane (SF) and its precursor GR are naturally occurring substances (phytochemicals) found in cruciferous vegetables. For many years now, the only reliable source of SF for clinical trials was our own broccoli sprout extract preparation. Whereas glucoraphanin has in recent years been made available in the supplement market by a number of manufacturers, SF has continued to elude commercialization. Now, with the introduction of Avmacol® to the dietary supplement market, a vetted, stable source of SF is available to clinical trial participants, and could be purchased by them or by others, even after their involvement in clinical studies ends. This is something that has not been possible with previous SF-rich broccoli sprout extract preparations.

The biologically active phytochemical in Avmacol® is glucoraphanin (GR; 4-methylsulfinylbutyl-glucosinolate), the precursor of sulforaphane (1-isothiocyanato-(4*R*)-(methylsulfinyl)butane). Sulforaphane (SF) is the aglycone breakdown product of the GR. The molecular formula of SF is $C_6H_{11}NOS_2$, and its molecular weight is 177.29 daltons. The structural formulae of GR and SF are shown in **Figure 1**. After ingestion and absorption, SF is conjugated with glutathione by glutathione-*S*-transferases (GSTs), then metabolized sequentially by γ-glutamyltranspeptidase (GTP), cysteinylglycinase (Cgase), and *N*- acetyltransferase (NAT) to form mercapturic acids, the predominant metabolites of SF found in urine (**Figure 2**) (26,27,31).

Figure 1. The Structural Formulae of Glucoraphanin and Sulforaphane

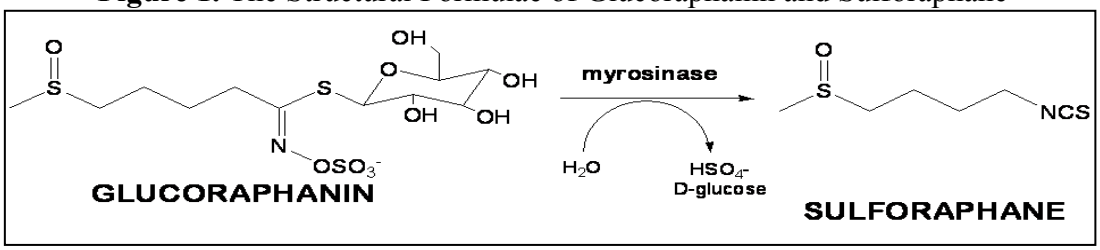
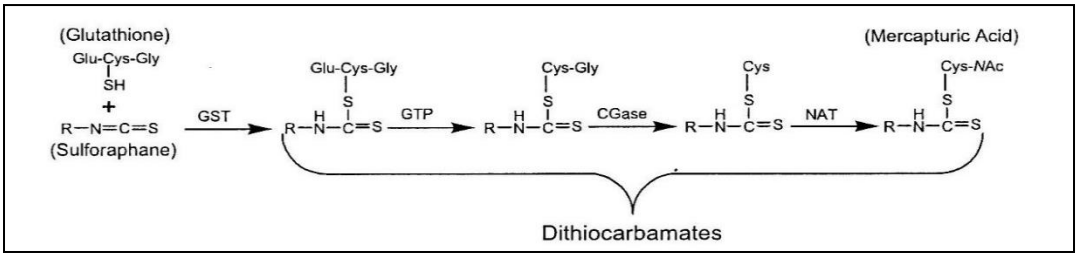


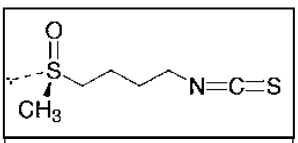
Figure 2. Metabolism of Glucoraphanin in Humans



Sulforaphane conjugates quickly accumulate in various types of cells in culture, reaching intracellular concentrations in the millimolar (mM) range (33). Intracellular accumulation is achieved through conjugation with cellular glutathione (34), a reaction that is accelerated by glutathione *S*-transferases (35), and the glutathione conjugate is then exported by a transporter-mediated mechanism (36). SF activates the Nrf-2 pathway resulting in elevated gene transcription via the Antioxidant-Response Element in the regulatory domain of its target genes (28).

4.1.3 Physical, Chemical, and Pharmaceutical Properties and Formulations

The active ingredient in Avmacol® (as in broccoli sprout extracts) is glucoraphanin (GR; 4-methylsulfinylbutyl-glucosinolate). The biologically active metabolite of this inert plant precursor, sulforaphane (SF; 1-isothiocyanato-4*R*-(methylsulfinyl)butane) is formed by the enzymatic action of myrosinase released when sprout tissues are damaged or chewed. SF conversion is maximized in broccoli sprout extracts through the addition of small amounts of myrosinase-containing daikon sprouts. Sulforaphane is an isothiocyanate. Its molecular formula is $C_6H_{11}NOS_2$, and its molecular weight is 177.29 daltons. The structural formula of SF is:



Sulforaphane

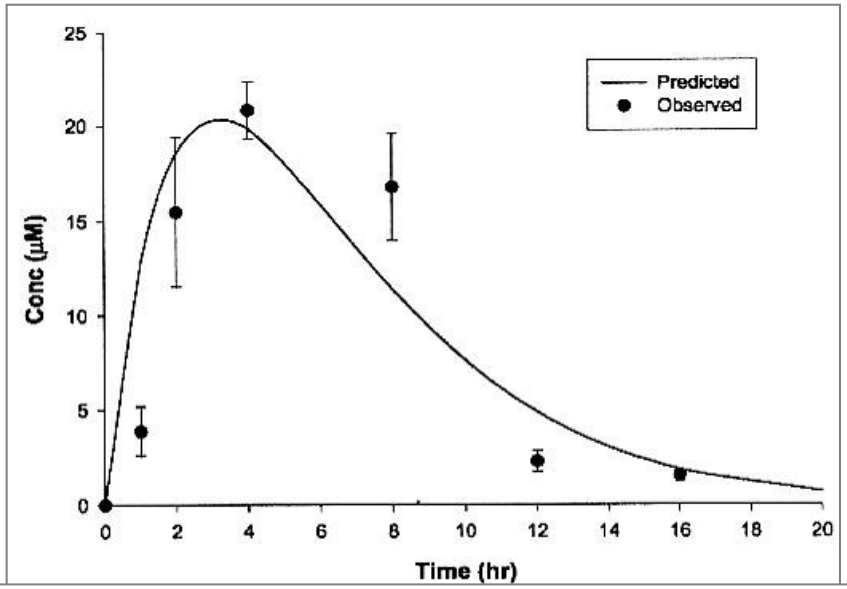


Figure 3. **Plasma concentrations of SF with respect to time after a 50 μmol oral dose of SF in rats with three animals per time point.** Results are mean ± S.D. The curve is the fitted plasma concentrations to a one-compartmental model with first-order absorption using WinNonlin (1).

Avmacol® is a proprietary supplement manufactured using industry standard tabulating and tablet-coating methods. In short, a broccoli seed and sprout extract (GR and myrosinase source) and excipients are pressed into tablets and then coated using industry standard technology.

4.1.4 Non-Clinical Pharmacology

4.1.4.1 Pharmacokinetics

Analysis of the pharmacokinetics of oral SF was performed using male Fischer rats receiving a single 50 μmol oral dose of SF. The average plasma concentrations of SF are illustrated in **Figure 3**. The plasma concentration of SF increased very rapidly, detectable at 1 h and peaking at 20.8 μM, 4 h after dosing. SF displayed fairly rapid absorption and an elimination half-life of 2.23 h (1).

4.1.4.2 Pharmacodynamics

Effects on gene expression

Hu *et al.* investigated the effects of two oral doses of 50 μmol of SF given at 0 and 24 hours on gene expression profiles by DNA microarray on male Fischer rat liver *in vivo*. 562 genes were found to be 2-fold up- or down-regulated at one or more time points (2, 4, 12, 24, 48 hours) compared to control. The most robust induction was of the metallothionein-like genes (MT-1/2 and MT-1a), which are considered important antioxidant genes, which increased up to 10-fold after SF dosing. Effects of this high

dose oral SF on selected cytochrome P450 genes were statistically significant with up-regulation of CYP 19, 3A3, 3A9, 1B1, 2B19, and 4B1 to 3.28-, 2.88-, 2.78-, 2.49-, 2.04-, and 2.05-times baseline, respectively. Cytochrome P450 2C29 and CYP1A1 were lowered to 20% and 72% of baseline levels, respectively (1).

Comblatt et al. investigated the effects of a single oral dose of 150 micromol of SF on gene and protein expression in Sprague Dawley rats. A maximal 12-fold induction of *NQO1* transcripts was observed in the mammary gland 12 h after dosing and there was significant induction as early as 2 h. A biphasic pattern of heme oxygenase-1 (*HO-1)* transcript induction was observed, with an initial peak at 2 h followed by a subsequent peak at 12 h. The minimal time to statistically significant *HO-1* induction was 1 h. A maximal 2.8-fold induction of NQO1 enzymatic activity in the mammary gland was observed 24 h after dosing with minimal time to statistically significant induction at 4 h (29).

Effects on enzyme expression

Murine studies of oral SF feeding have examined the effect on detoxication enzyme induction. When SF doses of 15 micromole (88.5 mg/kg/day) were administered to mice daily for 5 days by gavage, there was a 2.5-fold increase in hepatic NQO1 activity (37). Another murine study of SF administered intragastrically at a dose of 17 micromole (150 mg/kg/day) for 4 days showed 2.6 fold induction of mammary gland NQO1 activity, but hepatic levels of the enzyme were not significantly elevated (38). When SF (50 mg/kg/Day) was given daily for 7 Days by gavage to male Fischer rats the hepatic NQO1 activity increased significantly, but hepatic CYP 1A activity was not affected (37). Notably, no toxic effects were noted in these studies in which high doses of SF were administered.

Yoxall et al. (39) performed studies with oral SF in male Wistar rats. Using doses of 3 and 12 mg/kg daily for 10 days, they examined the effects of SF on cytochrome P450 enzymes. In these studies, SF exposure had no obvious adverse effect on the animals and body weight gain was unaffected. Treatment with SF elevated levels of CYP1A2 nearly doubling the level at the highest dose. However, the newly synthesized enzyme was not catalytically competent, at least partly due to SF metabolite binding to the enzyme rendering it metabolically inactive. Thus, the net effect was that activity of CYP1A2 was depressed. CYP2B expression decreased to 85% of control only at the higher SF dose. CYP3A2 expression decreased to 80% of control with SF treatment only at the higher dose. CYP2E1 was unaffected by SF.

4.1.4.3. Non-clinical Metabolism

Kassahun et al. (40) examined the biotransformation of SF in the rat by administering 50 mg of SF per kg to male Sprague-Dawley rats. Bile and urine were subsequently collected. In bile, five thiol conjugates were detected including glutathione (GSH) conjugates of SF, erucin (the sulfide analog of SF), the *N*-acetylcysteine (NAC) conjugates of SF, and the GSH conjugate of a desaturated metabolite of SF, suggesting oxidative metabolism of the parent compound. Fecal metabolites were not identified. NAC conjugates of SF were detected in the urine. Quantitative urine

determinations indicated that 60% of a single dose of SF is eliminated in 24 h as the NAC conjugate of SF. Thus, SF undergoes metabolism by S-oxide reduction and dehydrogenation. GSH conjugation is the major pathway by which the parent compound and its metabolites are eliminated in the rat.

4.1.4.4 Non-clinical Toxicology

A number of animal studies have investigated the biological effects and toxicology of oral SF-containing broccoli seeds and SF itself. While these investigations have examined a wide variety of endpoints and effects, they have demonstrated the overall *in vivo* safety and beneficial biological effects of broccoli seeds and SF. Animal studies are summarized below.

Myzak et al. (41) treated C57BL mice with 6 micromol SF/Day for 10 weeks with no adverse effect on body weight, hematocrit, or spleen weight. Taking into account species scaling factors, human consumption of 106 g of broccoli seeds daily would achieve daily SF intake similar to this murine study.

Jones et al. (42) treated male F344 rats with SF 50 mg/kg/Day (280 micromol / kg / Day) by gavage for five Days. The dose was selected based on reports of non-toxicity and efficacy in inducing cytoprotective enzymes in other model systems. During the study 2 of 10 animals in the SF treatment group died after feeding due to aspiration of the dose. On the sixth Day, after five Days of SF feeding, rats were sacrificed and organs examined. The SF was well tolerated by the animals and there was no apparent toxicity over the duration of the study. The SF feeding did not affect the relative weights of the prostate, kidneys, liver or bladder. Body weight was not affected compared to control animals, though there was an 8% decrease in body weight in the SF treated group compared to initial weight. The SF dose used in this study is >10-times greater than that achieved by the proposed BSP dosing for our protocol.

Zhang et al. (43) treated female Sprague-Dawley rats with BSP to study the effect on tissue GST and NQO1 and the urinary excretion levels of isothiocyanate (ITC) metabolites. These experiments included administration of BSP providing 40, 80, and 160 micromol isothiocyanate/kg body weight daily for 14 Days. None of the extract doses were associated with any sign of toxicity, with all rats in good health and body weight gain not significantly different among treatment and control groups. No gross abnormalities were detected at necropsy. No pathological changes were visible in rat bladder tissues when examined microscopically. BSP in the doses administered effectively induced GST and NQO1 enzyme expression in rat bladder, duodenum, and stomach in a dose-dependent fashion. Significant enzyme expression was induced in rat colon, kidney, and lungs at the higher dose of broccoli seed extract. Measurement of urinary ITC and metabolites by cyclocondensation assay showed extensive (70-78%) elimination of the ITC doses within 24 hours. ITCs are known to be metabolized *in vivo* mainly through the mercapturic acid pathway and to be excreted in the urine as NAC conjugates.

4.1.4.5 Mutagenicity

In a study in rats by Yoxall et al. conversion of 2-amino-3-methylimidazolo-[4,5-f] 18harmacod to mutagenic intermediates (Ames test) was reduced by treatment with SF at both 3 and 12 mg/kg/Day (17 and 68 micromol /kg/Day) doses (39).

4.1.5 Pharmacokinetics and Metabolism of Broccoli Seed Preparations

4.1.5.1 Broccoli Sprout Extracts

Ye *et al.* (44) conducted a human study with broccoli sprout extracts to provide information on plasma, serum, and erythrocyte concentrations of isothiocyanates (ITC) after ingestion of metered doses of ITC. Four healthy male human subjects were fed single doses of a SF-rich extract made from sprouted broccoli seeds, in which the glucosinolates had been hydrolyzed by daikon myrosinase to their cognate isothiocyanates, i.e. GR to SF. Cyclocondensation assays were performed on two occasions to verify the dose: first at the time of dose preparation and second near the time of feeding, with the two analyses within <5% agreement. The four subjects were fed a single dose of myrosinase-hydrolyzed extract of sprouted broccoli seeds containing 200 μmol of ITC (77% SF). Maximum urinary excretion rate of ITC and dithiocarbamates (DTC) metabolites of ITC, was attained between 1 and 1.5 hours in 3 subjects and between 0.5 and 1.0 hours in 1 subject. The cumulative 8 hour urinary excretion of the DTCs averaged 58.3 ± 2.8% of the dose. 72 hours after consumption, urinary excretion totaled 77.9 ± 6.4 % of the dose, suggesting the isothiocyanates did not react irreversibly with proteins or other macromolecules, but were rapidly converted to dithiocarbamate metabolites. The plasma and erythrocyte concentrations and urinary excretion rates plotted in **Figure 4** show that the pharmacokinetic behavior of the ITC was very similar in all 4 human subjects. ITC/DTC levels were detected in plasma/erythrocytes 15 minutes after dosing and the concentration rose to a maximum in the 1 hr sample of all subjects with a mean maximum of 2.00 ± 0.30 μmol/l; range: 1.62 – 2.27 micromol /l. Between 2 and 6 hours after dosing, plasma concentrations of ITC/DTC declined with first-order kinetics with a mean half-life of 1.77 ± 0.13 hours. The calculated renal clearance is 369 ± 53 ml/min, which is more than double the glomerular filtration rate for the subjects, suggesting tubular secretion plays a major role in the elimination process. The bioavailability of SF in humans is believed to be 60-90%. From these data, an apparent volume of distribution is 59.9 ± 7.0 liters which is consistent with ITC/DTC distribution in total body water (27,44). Despite the relatively short half-life, it is believed that SF has a longer duration of action since most of the induced phase 2 enzymes have half-lives measured in days. Thus, a high cellular concentration need not be maintained continuously (45).

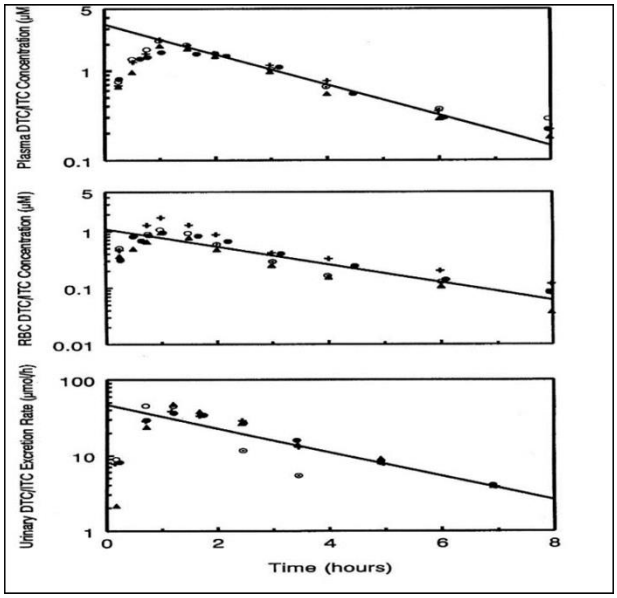


Figure 4. Dithiocarbamate Concentration Changes in Plasma, Erythrocytes, and Urinary Excretion Rates Over Time.

Semi-logarithmic plot of the time course of changes in the concentrations of dithiocarbamates in the plasma (upper panel) and erythrocytes (middle), as well as the rates of urinary excretion (lower) of four volunteers who received 197-210 μmol of broccoli

4.1.5.2 Broccoli sprout extract without daikon

We conducted a randomized, Phase Iia human study enrolling 200 subjects in Qidong, China. Healthy subjects were randomized to receive an extract of sprouted broccoli seeds ("tea") containing 400 μmol GR or placebo nightly for 2 weeks in an attempt to modulate carcinogen markers. Subjects were followed clinically for compliance and adverse events. Serum ALT and urinary dithiocarbamate (DTC) levels were assessed. DTC elimination varied up to 3-fold between participants but was consistent between doses within an individual, suggesting bioavailability of active isothiocyanates (SF) was variable among the population. Importantly, the GR in the extract was not treated with myrosinase and thus relied upon enteric bacterial flora for conversion to SF. Thus, the investigators speculate that differences in individual gastrointestinal flora may account for the variability. The broccoli seed tea was well tolerated. All 199 subjects completed all doses in the study without reporting adverse events. There was a strong inverse association between DTC excretion levels and excreted carcinogen biomarkers for aflatoxin and polyaromatic hydrocarbons, suggesting that ingestion of extract from sprouted broccoli seeds may favorably influence the metabolism pathway of foodborne and airborne toxicants (32).

4.1.5.3 Broccoli sprout extract with daikon

Egner et al. conducted a cross-over clinical trial in Qidong, China in 2009 to compare the bioavailability and tolerability of SF from beverages made with two sprouted broccoli seed powders: one glucoraphanin-rich (GR-rich) powder and the other sulforaphane-rich (SF-rich) powder. SF was either generated *in vivo* from the GR contained in the GR-rich powder by gut microflora or formed by *ex vivo* treatment of GR-rich powder with myrosinase from daikon (*Raphanus sativus*) seeds to generate SF-rich powder. Fifty healthy, eligible participants were requested to refrain from crucifer consumption and randomized into two treatment arms. The study design was

as follows: 5-Day run-in period, 7-Day administration of beverages, 5-Day washout period, and 7-Day administration of the opposite intervention. Isotope dilution mass spectrometry was used to measure levels of GR, SF and SF thiol conjugates in urine samples collected daily throughout the study. Bioavailability, as measured by urinary excretion of SF and its metabolites (in approximately 12 hour collections after dosing), was substantially greater with the SF (mean = 70%) than with GR-rich (mean = 5%) beverages. The primary urinary metabolite of following administration of either GR-rich or SF-rich powder was the acetylcysteine conjugate of SF. Inter-individual variability in excretion was considerably lower with the SF-rich than GR-rich beverage. Elimination rates were considerably slower with GR-rich beverages allowing for achievement of steady state dosing as opposed to bolus dosing with SF-rich beverages. Results from this trial led us to consider blends of SF and GR as SF-rich and GR-rich mixtures to achieve peak concentrations for activation of some targets and prolonged inhibition of others implicated in the protective actions of SF. The Avmacol® to be administered during this trial follows this pharmacokinetic principle, as the bioactive ingredients include broccoli seed powder, containing both GR and myrosinase which is responsible for consistent *in vivo* delivery of 40% of the GR content as SF (46).

4.1.5.4 Cruciferous Vegetables

Shapiro et al. conducted human studies on DTC excretion after cruciferous vegetable ingestion. Four healthy male volunteers were fed 250 grams of a cruciferous vegetable. Isothiocyanate levels were measured in each vegetable administered and contained 0.3 – 0.8 μmol/g fresh weight. Thus ITC dosing ranged from 75 – 200 micromol per vegetable dose. All 4 volunteers demonstrated a brisk rise in DTC urinary excretion after ingestion of crucifer doses. Excretion generally reached a peak in the first 8-h collection, was ≥ 80% complete in 24 h and returned to baseline by 72 h after dosing (27).

Lastly, Fowke *et al.* conducted an interventional trial in women over the age of 45 years. Participants attended a series of classes designed to facilitate the addition of cruciferous vegetables to the daily diet. Urine collection for ITC concentration was performed at baseline and during the 4-week intervention phase. The amount (grams) of cruciferous vegetables ingested was recorded during the baseline and intervention period. During the 4-week intervention phase, subjects consumed an average of 196 grams of cruciferous vegetables per Day (range 53-371 g). Group-average ITC excretion levels in urine followed the trend of vegetable intake with significant increases from Baseline to Intervention ($P < 0.01$) and a decrease from the Intervention to Post-intervention phase of the study ($P < 0.01$). No adverse reactions related to the dietary intervention were reported (47).

4.1.5.5 Broccoli preparations containing active myrosinase.

The bioavailability of a simple powder made by lyophilizing a boiling water extract of GR-rich broccoli sprouts (broccoli sprout extract; myrosinase de-activated by processing methodology), was compared with a variety of preparations containing

active myrosinase (BSdP - broccoli seed powder; BsdE - commercially prepared broccoli seed boiling water extracts that are spray-dried; FDBS - freeze-dried broccoli sprouts). Multiple sample matrices and modes of delivery showed that regardless of delivery method, providing active myrosinase as part of the preparation enhanced the bioavailability of SF and reduced the variability of conversion (GR to SF) which can be ascribed solely to the gut microbiota. Mean bioavailability of GR preparations lacking active myrosinase, as we have shown in previous studies, was roughly 10% of dose, whereas when active myrosinase was included in the dose, bioavailability increased to about 40%, see **Table 1**. Both within-subject and between subject variability was also reduced, and when hydrolysis was accomplished ex-vivo, prior to dosing, bioavailability was closer to 90% (48).

Table 1. Mean bioavailability of sulforaphane from broccoli sprout and seed preparations rich in glucoraphanin, as affected by matrix, mode of delivery, and activity of myrosinase on its substrate (48)

No.	Cohort	Matrix	Mode of delivery	Dose (μmol GR)	Mean availability (as % of dose)	Mean availability (as % of SF)	No. subjects enrolled[f]	No. subjects assessed
1	1	BSE[a]	Dissolved in water	50	9.4	−	5	5
2	2	BSE	In gelcaps	69	12.8	−	20	16
3	2	BSE	In gelcaps	230	8.3	−	20	18
4	3	BSdE[b]	In gelcaps	69	11.2	−	20	17
5	3	BSdE	In gelcaps	230	9.7	−	20	19
6	4	BSdP[c]	In standard gelcaps	100	32.6	−	5	4
7	4	BSdP		100	31.7	−	5	4
8	4	BSdP		100	44.1	−	5	4
9	5	FDBS[d]		100	35.1	−	5	5
10	5	FDBS	In acid-resistant gelcaps	100	32.7	−	5	5
11	6	FDBS	Pre-hydrolyzed[e] in pineapple-lime juice	50	33.6	n.d.	5	5
12	6	FDBS		50	48.4	97.6	5	5
13	6	FDBS		50	40.2	91.4	5	4
14	6	FDBS		100	41.8	84.3	5	4
15	6	FDBS		200	40.9	83.9	5	4

[a] Broccoli Sprout Extract; [b] Broccoli Seed Extract produced commercially as OncoPLEX™ (from Xymogen); [c] Broccoli Seed Powder (with active myrosinase); [d] Freeze-Dried Broccoli Sprouts (with active myrosinase); [e] Myrosinase-converted for 10' at room temperature in juice; [f] A subject pool of 5 volunteers participated in most tests described (numbers 1-15 above). They were augmented with another 15 volunteers for tests numbered 2-5 above.

4.1.6 Safety and Efficacy

Cruciferous vegetables, including broccoli seeds and broccoli seed sprouts, are generally regarded as safe and are regular dietary components in many regions of the world. Previous estimates of the daily dietary intake of cruciferous vegetables vary regionally, averaging 40 g/Day in Singapore (49), 11 g/Day in the United States (50), 16 g/Day in Canada (51), 30 g/Day in the UK (52), and 112 g/Day in Japan (53).

4.1.6.1 Broccoli Sprout

A number of small pilot clinical studies have evaluated the effect of broccoli sprouts on antioxidant endpoints:

Murashima and colleagues reported in 2004 on the elevation of multiple biomarkers of oxidative stress following a one-week course (6 young male and 6 young female smokers) of 100 g/d of fresh broccoli sprouts (54). Plasma markers were measured before and after treatment. Treatment produced decreases in serum total cholesterol, LDL cholesterol, coenzyme Q_{10}, plasma 23 harmaco and phosphatidylcholine hydroperoxide, and urinary 8-isoprostane, and 8-OhdG. Increases in $CoQ_{10}H_2/CoQ_{10}$ ratio, and HDL cholesterol were observed. Blood lymphocyte markers, natural killer cell activity, triacylglycerol, urea nitrogen, uric acid, AST, ALT, γ-GPT, and plasma amino acids were also measured and there were no before- to after-treatment differences in any of these biomarkers.

In 2009, a group at UCLA reported on the ability of orally administered broccoli sprout homogenates (BSH) to increase phase 2 antioxidant enzymes in the upper airway (55). After feeding 57 subjects doses of BSH ranging from 25 to 200 grams on 3 separate days, followed on the 4th day by blood and nasal lavage collection, there was a dramatic and dose-dependent increase in phase 2 enzyme expression (mRNA for GSTM1, GSTP1, HO-1, and NQO1). Induction of individual phase 2 enzymes in nasal lavage cells was strongly correlated. No serious adverse events were reported, and dose intolerance or side effects of broccoli sprouts were not observed, and mild, digestive effects are presented in the report.

Christiansen et al. (56) report that ingestion of broccoli sprouts does not improve endothelial function in human beings with hypertension (n = 40 hypertensive, non-diabetic subjects with cholesterol in the normal range). Subjects were fed 10 g of dried broccoli sprouts for 4 weeks, and their blood pressure, endothelial function (measured by flow-mediated dilation), and blood samples were obtained every other week. Glucoraphanin content of the sprouts was measured, and equated to a dose of 259 μmol GR/day – a reasonable level, but in light of our findings perhaps not high enough to expect a measurable effect due to low conversion of GR to SF by the intestinal microflora of individuals.

4.1.6.2 Broccoli Sprout Preparations (BSPs) and Extracts (BSEs)

Shapiro et al. (31) conducted a double-blind, placebo controlled, randomized, Phase I study of two BSPs, beverages containing SF-rich or GR-rich powder made from sprouted broccoli seeds, in healthy individuals to determine the safety and tolerance of repeated oral administration. Twelve healthy human volunteers received doses of BSP every 8 hours for 7 Days (total 21 doses) while undergoing clinical evaluation and a battery of laboratory tests. Doses used in 3 cohorts of 3 subjects and 1 control were 25 μmol glucosinolates, 100 μmol glucosinolates, and 25 μmol isothiocyanates, respectively. Thus subjects received 75 – 300 μmol glucosinolates daily, equivalent to 12 - 50 g of fresh broccoli seeds, or 75 μmol isothiocyanate. No clinical adverse events were reported. With regard to laboratory testing, samples were obtained 6 times during the 19 Day study and included evaluation of the blood for the following: CBC with differential, reticulocyte count, PT, PTT, BUN, creatinine, Na, K, CO_2, Cl, glucose, albumin, direct/total bilirubin, alkaline phosphatase, AST, ALT, GGT, T3, T4, TSH. Urinalysis was performed for urine creatinine, and urine dithiocarbamates

(DTC). Two of 12 individuals (both receiving active preparations) showed an increase in plasma ALT exceeding the upper limit of normal with one meeting criteria for a Grade 1 toxicity. Notably, ALT levels rose for all subjects during the course of the study including placebo-treated subjects. Plasma AST levels rose above normal on Day 19 for 2 of 12 subjects. They were released from the inpatient portion of the study on Day 17 and their post-discharge activities (i.e., possible alcohol intake) could not be ascertained. Monitoring of TSH levels demonstrated that in 3 of 12 subjects (2 active treatment, 1 placebo), TSH levels exceeded the upper limit of normal during or after the dosing period. Notably, TSH levels rose for 11 of 12 subjects during the first 6 Days of hospitalization before broccoli seed administration was begun. TSH increases were not associated with any clinical symptoms or abnormalities of T3 or T4. Evaluation by 2 independent endocrinologists determined that the changes in TSH were mild and reversible and did not pose an obstacle to further studies with administration of BSPs. No other significant laboratory abnormalities occurred. Thus, this Phase I safety study in healthy volunteers revealed no evidence of systematic, clinically significant adverse effects that could be attributed to the administration of repeated doses of broccoli sprout extracts containing SF or GR (31).

Cornblatt *et al.* (29) conducted a study to assess the bioavailability of SF-rich broccoli sprout extract in human breast tissue. In this proof-of-principle study, 8 women undergoing elective mammoplasty consumed a preparation containing 200 μmol of SF approximately 50 minutes prior to surgery. The extract was well-tolerated without any adverse events or complications. SF metabolite levels measured by cyclocondensation reaction as dithiocarbamates were used to determine SF distribution. Mean post-dose plasma dithiocarbamate (DTC) level was 0.92 ± 0.72 μM, and mean epithelial/stromal enriched breast tissue DTC concentration was 1.45 ± 1.12 and 2.00 ± 1.95 picomol/mg tissue for right and left breast respectively. In addition, the investigators were able to measure NQO1 and HO-1 transcripts in the human breast tissue, demonstrating the feasibility of assessing a 24harmacodynamics action of SF in these tissues (29).

In an ongoing randomized clinical trial at Johns Hopkins, 21 women completed a 10 day intervention of broccoli sprout extract or placebo (mango juice). There were only three grade 1 mild gastrointestinal adverse events reported and no significant changes (1.5 times or greater) in bloods (comprehensive metabolic panel, full blood count, coagulation panel and thyroid tests), taken pre and post intervention (personal communication K. Visvanathan).

In the cross over trial conducted by Egner et al (46), 2 of the fifty participants randomized to receive the SR-rich beverage (150 μmol/day) during either the first or second wave complained of nausea or bitter taste and dropped out of the study. Serum chemistry studies conducted on samples obtained after the last of seven daily doses of SF did not present any abnormal values. This study suggests that 150 μmol SF/day, approximates the maximum tolerated dose.

A very recent report from Iran, describes the effect of oral broccoli sprouts on a variety of oxidative stress biomarkers in a double-blind, placebo-controlled, randomized controlled trial, in type 2 diabetes patients. Eighty-one patients were randomly assigned to one of three treatment groups for 4 weeks. They received either 10 g/day, or 5 g/day broccoli sprout powder (BSP), or a placebo of cornstarch and chlorophyll (57). The authors report that consumption of BSP resulted in a significant decrease in malondialdehyde, oxidized LDL cholesterol, and "oxidative stress index", and a significant increase in total serum antioxidant capacity. They found no significant effect on "total antioxidant status" or fasting blood glucose. Since this study employed an over-the-counter broccoli sprout supplement and the authors did not make independent determinations of its SF content, and since the fasting blood glucose values for the three groups were not closely matched, there is substantial concern about the robustness of reported results, although there do appear to be clear trends for difference.

In a very recent study of men with recurrent prostate cancer at the Knight Cancer Institute in Portland, Oregon, treatment with 200 μmol of sulforaphane per day for 20 weeks produced no major side effects and adverse events were mostly grade 1 gastrointestinal (58).

Dr. Kensler and his colleagues conducted a randomized phase II trial from October 2011 to January 2012, in which 267 healthy volunteers in Qidong City, China (a region with extremely high liver cancer prevalence) received 84 consecutive daily doses of a broccoli sprout beverage containing 600 μmol of glucoraphanin and 40 μmol of sulforaphane, or an indistinguishable placebo (17). The study participants comprised 136 treated with broccoli sprout beverage and 131 placebo controls. Of the 267 participants completing the study, 50% consumed all assigned doses, and the remaining participants consumed at least 80 of the 84 doses. Extensive blood chemistries at the termination of the study showed that the means of 13 analytes were identical between treated and control groups, and that specifically no abnormalities were detected in BUN, creatinine, and transaminases (ALT and AST). Three individuals, 2 in the placebo arm and 1 in the treated arm, had slightly elevated total bilirubin (1.1, 1.6, 1.8 mg/dL) and two individuals, 1 each in placebo and treated arms, had slightly elevated direct bilirubin (0.5, 0.6 mg/dL levels).

4.1.7 Rationale for Selected Doses

We will use doses of Avmacol® that will provide equivalent concentrations of SF as found in our most recent trials of various BSP preparations. Four Avmacol® tablets twice daily will provide an internal dose of 120 micromole SF per day. The dose selection is based on the results of our previous clinical trials (NCT02023931) with the maximum tolerable dose and minimal toxicity effect to maximize the biological effect (17,59).

This dose level represents bioactive and tolerable doses in healthy volunteer studies. This dose was found to be tolerable and without safety compromise with no grade 2 or higher toxicities reported. Although bad taste was the main complaint by study subjects consuming beverage made from SF-rich sprouted broccoli seed powder at 150 μmol/day, the encapsulation of Avmacol® is expected to mask any issues with

taste. This dose is expected to achieve a target level of NRF2 pathway activation based on a previous healthy volunteer study (59). The twice-daily dosing schedule will provide a steady level of in vivo SF over the 24 hr a day, as shown in a recent report (60).

4.2 Rational for Proposed Study in High-Risk Former Smokers

It is critical to carefully define entry criteria for any lung cancer prevention trial. Study subjects must be of high enough risk that either lung cancer or intermediate endpoints will be found as frequently as possible. We have created a unique high-risk cohort of approximately 3,600 current and former smokers, the Pittsburgh Lung Screening Study (PluSS). At baseline, we administered a risk factor questionnaire, tested pulmonary function, assessed low-dose computed tomography (LDCT) for emphysema severity, created a biorepository (serum, plasma, and DNA), and implemented procedures to ascertain and characterize new lung cancer diagnoses. At enrollment, PluSS participants had a mean age of 60 years. Among the participants, 49% were women; 93% whites, 5.5% blacks, and 1.7% other race/ethnicity groups; 60% current smokers and 40% former smokers with a median 47 pack-years of smoking history (61). The PluSS participants resembled the national representative sample of current and former smokers who are at high risk for lung cancer. For example, the National Lung Screening Trial (NLST) enrolled more than 53,000 persons at high risk for lung cancer at 33 medical centers across the US. The mean age was 61 years. Forty-one % were women; 91% whites, 4% blacks, and 5% other race/ethnicity groups; 48% current smokers and 52% former smokers who quit smoking within the previous 15 years; a median 48 pack-years of smoking history (62).

This unique cohort has contributed many scientific discoveries in risk biomarkers of lung cancer (61,63-72). For example, the presence of chronic obstructive pulmonary disease (COPD) significantly increased lung cancer risk (61,66). Compared with smokers without COPD, smokers with COPD measured as the Global Initiative for Chronic Obstructive Lung Disease (GOLD) II or higher or Forced Expiratory Volume in the first second (FEV1) < 80 % had more than doubled risk of developing lung cancer after controlling for smoking intensity and duration (**Table 2**).

Table 2. Hazard ratios (HRs) of lung cancer associated with baseline airflow obstruction and radiographic emphysema in the Pittsburgh Lung Screening Study

	Cases	Noncases	Adj. HR (95% CI)*
Airflow obstruction			
None	32	2053	1.00
GOLD I	16	477	1.66 (0.89-3.11)
GOLD II	36	792	2.11 (1.27-3.49)
GOLD III-IV	15	217	2.86 (1.48-5.53)
P for trend			*0.005*
Radiographic emphysema			
None	24	2068	1.00
Trace	22	663	2.58 (1.43-4.66)
Mild	37	493	5.04 (2.94-8.62)
Moderate/severe	16	315	3.20 (1.65-6.23)
P for trend			*<0.0001*

* Adjusted for age, sex, years of cigarette smoking, and number of cigarettes per day.

The presence of mild, moderate or severe radiographic emphysema on the LDCT scans at baseline was significantly associated with a 3- to 5-fold increased risk of developing lung cancer compared to smokers without emphysema (61).

We will recruit subjects for the present study from the existing PluSS participants who had mild, moderate or severe emphysema or GOLD II-IV COPD. Among the original PluSS participants who, as of 12/31/2015, were <75 years old, alive, free of cancer, and had quit smoking within the past 15 years, 356 had GOLD II-IV COPD (with or without emphysema), and additional 167 with mild, moderate or severe emphysema (without COPD). All these 523 subjects are potentially eligible for the proposed study. Based on our previous experience in the Iloprost study (22), which used a very similar protocol as the one proposed here, there was a 20% recruitment rate. Thus, we expect to fulfill our recruitment goal of at least 72 former smokers into the study within the first 3 years of the proposed study. With a 20% dropout rate based on our previous experience (22), we expect to have 60 study subjects who will complete the entire course of the trial.

If the proposed chemopreventive agent is effective, it will bring beneficial effect for study participants on reducing their risk of lung cancer. After the end of the study, all study participants can conduct their oral intake of Avmacol® since this dietary supplement is relatively cheap and commercially available.

5. TREATMENT PLAN AND STUDY PROCEDURES

5.1 Pre-Treatment Evaluation

Pretreatment tests should be performed within 90 days prior to the randomization.

1) Signed informed consent
2) Questionnaire data collection on patient's characteristics and smoking history.
3) Physical exam including height, weight, vitals and SWOG performance status.
4) FEV1/FVC
5) Blood oxygen saturation
6) Expired Carbon Monoxide
7) Laboratory analysis including CBC and serum chemistries such as total bilirubin, AST, ALT, BUN, creatinine, .
8) Baseline bronchoscopy: Prior to randomization, all participants will undergo a bronchoscopy at the Endoscopy Suite at the UPMC Shadyside Hospital. The procedure will be carried out by Dr. Wilson (Co-PI) using Narrow Band Imaging (NBI) white-light bronchoscopy. The patient's nose, throat, vocal cords and windpipe are sprayed with Lidocaine (numbing medicine) to help keep patient from coughing and to numb patient's airways. During the procedure, Dr. Wilson will perform a complete airway examination including visualization of the vocal cords, trachea, main carina, and orifices of the sub-segmental bronchi to the extent that it is visible without causing trauma to the bronchial wall. Biopsy samples will be collected at the pre-determined sites. Some of these biopsies will be used for the pathological examination. Any patients with lung cancer or carcinoma in situ will be ineligible for the present study. We will inform subjects the abnormal biopsy results and provide appropriate counseling from qualified medical personnel.

Since the clinical significance of moderate or severe dysplasia is unknown and no effective treatment available, we will not conduct additional clinical procedure or intervention on these patients beyond routine follow-up and care. The patients with dysplasia will be monitored at each clinical visit (once every three months during the treatment period) according to the study protocol. At the end of study period, another biopsy at the same site of initial locations that show dysplasia will be performed to evaluate the change (progress, regress or no change) of these lesions detected at baseline biopsy. This is one of the primary outcome measures for the proposed study. After the completion of the study, the patients will continue to receive routine medical care including annual CT screening if eligible.

5.2 Randomization

Eligible subjects will be randomly assigned to either Avmacol® or placebo arm with a 50%-50% chance. Each subject will be given three bottles of tablets at month 0 and at trimonthly clinical visits. Each bottle contains 250 tablets that will be sufficient for one month supply.

5.3 Treatment Plan

5.3.1 Nutraceutical Information

Please see the Avmacol® Nutraceutical Information Guide for detailed information on manufacturing and quality assurance (Appendix 11).

For this study, capsules will be shipped from Nutramax to the University of Pittsburgh as follows:

c/o Brian M. Miller, PharmD
Director, Investigational Drug Service
UPMC Hillman Cancer Center
Ground Floor, AG40.3
5115 Centre Avenue
Pittsburgh, PA 15232
Avmacol® capsules will be stored at room temperature in the Investigational Drug Pharmacy at the UPMC Hillman Cancer Center. After dispensing, they may be stored at room temperature at the participant's home.

5.3.2 Dose

Following randomization, subjects will begin to take four study tablets (Avmacol or placebo) in the morning with breakfast and four tablets in the evening with dinner. Each tablet contains 15 mg of glucoraphanin (GR), but contains no SF. Thus the daily ingested dose is 120 mg or 274.29 micromole GR (molar mass 437.49). Based on our most recently completed bioavailability studies in humans (48), the internal conversion rate of GR to SF in standard gelcaps (the same formula for the present study) was 44% (see Table 1 – Cohort 5 under Section 5.5.1 Pharmacokinetics and Metabolism in Humans on page 23). Thus the 8 Avmacol tablets provide a daily internal dose of approxi 120 micromole SF (274,29 x 0.44 = 120.68 micromole SF).

5.3.3 Treatment Duration

The Avmacol tablets will be administered to study participants for a total duration of 12 months. Data regarding the duration of SF administration on altering cellular

markers in human bronchial tissues are lacking. In a recent phase 2 clinical trial lasting 2-8 weeks, Atwell *et al*. evaluated the effect of SF on level of Ki-67 LI in breast benign tissue in women who underwent diagnostic biopsy and surgical treatment for breast cancer. The daily dose of 513 micromole gluroraphanin (SF precursor) significantly reduced Ki-67 expression in the benign breast tissue (P<0.01) (16). We note the conversion rate for this formulation of glucoraphanin to SF was not determined by the authors (16). It is likely lower than our Avmacol formulation: typically 5% in the absence of myrosinase. Our Avmacol tablets provide optimal internal dose of SF on apoptosis/proliferation markers, by pushing the duration of SF administration to 12 months.

5.3.4 Recording of Daily Intake Dose

To track the daily intake of study tablets, study participant will be required to keep a diary to record the number of tablet taken and the times of the day when the test tablets are taken (See Appendix 7. Log Form for Daily Intake of Test Tablets).

5.3.4 Criteria for Removal from Study

Given the absence of dose-limiting toxicities described in prior clinical trials, clinically significant toxicity is not expected from exposure to Avmacol® tablets. However, if a participant shows symptoms or signs of Grade ≥2 toxicity attributable to Avmacol®, the participant will be withdrawn from the trial and the study supplement will be discontinued. Follow-up will be maintained until the abnormality is resolved.

Participants maintain the right to leave the trial of their own volition at any point.

5.4 Dosing Delays/Dose Modifications

There are no plans for a dose modification in the study.

5.5 Clinical Visits

Besides two clinical visits in stage 1 of the study for eligibility screening, all eligibility study participants will begin taking the study tablets at visit 1 of stage 2 of the study and return to the study clinic at the end of months 3, 6, 9, and 12 (the end of treatment period). At each clinical visit, the clinical research nurse/assistant will conduct the following:

1) Exam the subject's diary for dosing and timing of intake of test tablets, and keep the diary in subject's folder.
2) Count and record the remaining tablets given.
3) Provide new study tablets that will supply the next 3 months.
4) Study participant will turn in the self-administered questionnaire for recording his/her intake of cruciferous vegetables in the past 3 months using a dietary frequency questionnaire (Appendix 5).
5) Collect blood, spot urine and nasal brushing samples
6) Conduct pulmonary function test: FEV1/FVC
7) Collect safety and toxicity measures at each clinical visit (once every 3 months) during the 12-month treatment period:
   a) Collect adverse events (AEs) using the AE Recording Form (Appendix 8)

b) Conduct physical examination including body weigh, height, and vitals by a registered nurse and record the results in the patient's study file

c) Laboratory evaluation: CBC with platelets and differential, chemistry panel including liver function testing.

8) Only at the end of treatment period, bronchoscopy will be performed on the patient using the same procedure as described above in 5.1(8) and bronchial biopsies and lavage samples collected (stage 2- visit 5).

5.6 Withdrawal from the Protocol

1) Unacceptable toxicity
2) Development of cancer
3) Patient refusal
4) Noncompliance
5) Death

5.7 Off Study Date

The "Off Study" date will be the date of the last clinical visit, i.e., stage 2 – visit 6 after the second bronchoscopy. If the patient withdraws from the study earlier, but agrees to the post-treatment follow-up, they will be contacted via telephone one month after the original off-study date.

5.8 Post-Treatment Follow-Up

1) Study participants will be contacted by telephone at the end of first month post-treatment for general health

2) All study participants will have routine follow-up as we do for the PluSS study participants through mails on an annual basis, to fill out a follow-up questionnaire for the diagnosis of lung cancer or any other cancer or vital status (filled out by the next-of-the-kin).

6. STUDY CALENDAR

		Stage 1		Stage 2 (after randomization)						Post-treatment	
Clinical visit	Pre-clinical	#1	#2	#1	#2	#3	#4	#5	#6	Phone	Mail
Targeted Date				Day 0*	Day 90± 14	Day 180 ±14	Day 270±14	Day 360± 14	Day 367± 10	1 mo. after	1 yr. after
Eligibility Screening Form	Phone Interview										
MAIL OUT FORMS											
Locator Form	(mail)	(Rec)									
Baseline **Q**uestionnaire	(mail)	(Rec)					(mail)		(Rec)		
Cruciferous **V**egetable intake form (compliance)				X	X	X	X		X		
SIGN CONSENT FORM	(PluSS PID)	X									
Assign subject ID (SF-Seq #)†		SF-001 SF-002 …									
PHYSICAL EXAM											
Vitals, weight & height		X		X	X	X	X		X		
CO test		X		X	X	X	X		X		
O2 test		X									
Spirometry (PFT)		X		X	X	X	X		X		
Urinary Pregnancy Test		X									
BLOOD (for Screening or AE Monitoring)											
CBC/platelets		X			X	X	X		X		
Chemistry panel & LFT		X			X	X	X		X		
BRONCHOSCOPY											
Bronchial biopsy			X					X			
Bronchial brushing			X					X			
Bronchoalveolar lavage			X					X			
INCLUSION/EXCLUSION FORM	(X)	(X)	(X)								
TREATMENT ASSIGNMENT (TA-Seq #)‡				TA-001 TA-002 …							
Provide study tablets (for 3 months)				X	X	X	X				
Pill count in returned bottles					X	X	X		X		
RESEARCH SAMPLES											
Blood				X	X	X	X		X		
Nasal brushing				X	X	X	X		X		
Spot urine				X	X	X	X		X		
AE ASSESSMENT FORM				X	X	X	X		X		
COMPENSATION		$50	$500	$50	$50	$50	$50	$500	$500		
Provide parking ticket		X	X	X	X	X	X	X	X		
FOLLOW-UP											
General health										X	X
Lung cancer										X	X
Any cancer										X	X

* Day 0 is when the subject is given a treatment assignment number.

† Study subject ID is assigned right after the consent form is signed by a potential eligible subject. This number will be used for the entire study and all forms and sample collection sheets.

‡ Treatment assignment number is TA-sequential # of subjects entering into stage 2 of the study. the TA # randomly determines the subject's treatment arm..

7. ADVERSE EVENTS: LIST AND REPORTING REQUIREMENTS

7.1 Adverse Events and Risks

Adverse event (AE) monitoring and reporting is a routine part of every clinical trial. The safety reports will be coded using current versions of Medical Dictionary for Regulatory Activities (MedDRA). Toxicity severity will be reported using National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE) term and grade: The descriptions and grading scales found in the revised NCI CTCAE version 4.0 will be utilized for adverse event reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 4.0. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site (http://ctep.cancer.gov/protocolDevelopment/adverse_effects.htm).

7.1.1 Adverse Events for Broccoli Sprout Preparation Avmacol®

There is no recognized risk from administration of Avmacol® tablets. The previous studies using broccoli sprout beverages in humans reported that the most common adverse events (AEs) or complaint by study participants was bad taste. As a tablet formulation, Avmacol will overcome the bad taste. Other expected AEs in gastrointenstinal system are nausea and flatulence. Table 3 summarizes reported AEs in previous studies involved more than 500 study subjects.

There may be adverse events or side effects that are currently unknown. Everyone taking part in the study will be monitored carefully for any side effects, including routine physical examinations, complete blood count (CBC), blood chemistry including liver function test, and food intolerances. All AEs and toxicity measures will be evaluated as one of the objective of the present study.

Table 3. Summary of Reported Adverse Events (AEs) in Studies Using Sulforphane or Sulforaphane Precursor Glucoraphanin in Humans

Study (reference)*	Design	Subjects	Dose*	Treatment duration	AEs
Kensler et al(32)	Randomized placebo-controlled phase 2 study	200 healthy volunteers	400 µmol glucoraphanin (GR)/day in hot water infusion of broccoli sprouts	14 days	No reported AEs
Shapiro et al(31)	Randomized placebo controlled phase I study	12 healthy volunteers	75-100 µmol GR/day	7 days	No clinical AEs; No abnormal chemistry test results. 2/12 subjects had ALT >1x ULN (grade 1 toxicity). Note: ALT level rose for all subjects including placebo-treated ones
Corblatt et al(29)	Single arm study	8 women undergoing elective mammoplasty	150 µmol SF	Single dose	Well tolerated, no any AEs or complications
Egner et al(46)	Randomized placebo-controlled phase 2 study	50 healthy volunteers	Broccoli sprout extract (BSE) beverage: 800 µmol GR/day	7 days	No AEs observed
Egner et al(17)	Randomized placebo-controlled phase 2 study	267 healthy volunteers	BSE beverage: 600 µmol GR + 40 µmol SF per day	84 days	10 participants (6 in BSE and 4 in placebo arm) reported grade 1 AEs: bad taste and mild stomach discomfort. One subject (in BSE) reported vomiting. Clinical values of 13 analytes of blood chemistry and liver function test for all participants at the last day of the intervention were all normal.
Atwell et al(16)	Randomized double-blind placebo-controlled phase 2 study	54 women with abnormal mammograms and scheduled for breast biopsy	BSE BroccoMax: 180 mg [= 518 µmol] GR/day	2-8 weeks	No difference in AEs between two groups AEs / BroccoMax / Placebo Bloating 5/27 5/27 Gas/Flatulence 1/27 4/27 Diarrhea 1/27 2/27 Others 5/27 7/27 All 8/27 9/27
Bauman et al(59)	Singe arm study	10 healthy volunteers	BSE beverage: R1: oral 600 µmol GR/day R2: oral 150 µmol SF/day R3: oral rinse 150 µmol SF for 5 minutes, and expectorated	Three 5-day intervention	No AEs reported

7.1.2 Risks Associated with Study Procedures

7.1.2.1 Bronchoscopy

Bronchoscopy is very safe, but no procedure is entirely free of potential risk. The following is a description of the risks of the procedure. In recent publications, the incidence of serious complications was less than 0.05% (5 in 10,000) in over 23,000 bronchoscopies. Local discomfort (coughing, gagging and soreness of your nose and throat) are frequently unavoidable but can be greatly diminished with adequate anesthesia. Bronchitis is common after bronchoscopy and may resolve spontaneously or with antibiotic treatment. Nosebleed, wheezing (about 1% chance), a decrease in blood oxygen content of approximately 10% for approximately one half hour, low grade fever (less than a 5% chance), and lung infection (pneumonia) in less than 0.1% and slight expectoration of small flecks of blood for 24 hours after the procedure (as a result of minor trauma to the bronchial lining) may occur. More serious complications such as major bleeding, lung collapse, vocal cord and bronchial spasm, and cardiac irregularity have been reported but are very rare. Since the patient will be medicated for this procedure they will need to make arrangements for someone to drive them home.

A Bronchoscopy Patient Communication Log Form is attached (Appendix 9) to follow-up the patients after the bronchoscopy is performed. Patients are called within 24 hrs., 72 hrs., and instructed to call within 30 days, if febrile, or with increased sputum production, or increasing shortness of breath or any perceived deterioration of respiratory condition.

7.1.2.2 IV Infusion During Bronchoscopy

An intravenous needle will be placed in the subject's arm for the infusion of fluids. The subject can expect to experience some pain at the moment the needle goes into the arm. In addition to this momentary pain, there will be the minor discomfort of having the needle taped in the arm. In about 10 percent of cases small amount of bleeding under the skin will produce a bruise (hematoma). The risk of temporary clotting of the vein is about 1 percent, while the risk of infection of a hematoma or significant external blood loss is less than one in 1,000. In order to minimize these discomforts, only trained doctors and technicians will be permitted to place the intravenous line.

7.1.2.3 Venous Blood Collection

An intravenous needle will be used to collect blood samples from the subject's arm. The collected blood samples will be used for clinical blood tests (CBC and chemistry) for subject's general health and for research use. The subject can expect to experience some pain at the moment the needle goes into the arm. In about 1-2 percent of cases small amount of bleeding under the skin will produce a bruise (hematoma). The risk of temporary clotting of the vein is about less than one percent, while the risk of infection of a hematoma or significant external blood loss is less than one in 1,000.

In order to minimize these discomforts, only trained clinical research nurses or technicians will be permitted to place the intravenous line.

7.1.2.4 Nasal brushing

Nasal cell scraping may be associated with pressure, pain or minor bleeding. The patient may feel some discomfort and nasal sore after nasal brushings. Nasal bleedings are minor and can be stopped after holding the nose for a few minutes.

7.1.3 Violation of Privacy and Loss of Confidentiality

Violation of privacy and loss of confidentiality are both risks to which research participants are exposed. The possibility of these risks increases when protected health information is collected.

7.2 Definitions of Adverse Events

Adverse event: Any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related.

Life-threatening adverse event or *life-threatening suspected adverse reaction:* An adverse event or suspected adverse reaction is considered "life-threatening" if, in the view of either the investigator or sponsor, its occurrence places the patient or subject at immediate risk of death. It does not include an adverse event or suspected adverse reaction that, had it occurred in a more severe form, might have caused death.

Serious adverse event or *serious suspected adverse reaction:* An adverse event or suspected adverse reaction is considered "serious" if, in the view of either the investigator or sponsor, it results in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

Suspected adverse reaction: Any adverse event for which there is a reasonable possibility that the drug caused the adverse event. For the purposes of IND safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the drug and the adverse event. Suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

Unexpected adverse event or *unexpected suspected adverse reaction:* An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or, if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended. For example, under this definition, hepatic necrosis would be unexpected (by virtue of greater severity) if the investigator brochure referred only to elevated hepatic enzymes or hepatitis. Similarly, cerebral thromboembolism and cerebral vasculitis would be unexpected (by virtue of greater specificity) if the investigator brochure listed only cerebral vascular accidents. "Unexpected," as used in this definition, also refers to adverse events or suspected adverse reactions that are mentioned in the investigator brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation.

7.3 Reporting Adverse Events to the Responsible IRB

In accordance with applicable policies of the University of Pittsburgh Institutional Review Board (IRB), the Sponsor-Investigator will report, to the IRB, any observed or volunteered adverse event that is determined to be 1) *associated with the investigational drug or study treatment(s)*; 2) *serious*; and 3) *unexpected*. Adverse event reports will be submitted to the IRB in accordance with the respective IRB procedures.

Applicable adverse events will be reported to the IRB as soon as possible and, in no event, later than 10 calendar days following the sponsor-investigator's receipt of the respective information. Adverse events which are 1) *associated with the investigational drug or study treatment(s)*; 2) *fatal or life-threatening*; and 3) *unexpected* will be reported to the IRB within 24 hours of the Sponsor-Investigator's receipt of the respective information.

Follow-up information to a reported adverse event will be submitted to the IRB as soon as the relevant information is available. If the results of the Sponsor-Investigator's follow-up investigation show that an adverse event that was initially determined to not require reporting to the IRB does, in fact, meet the requirements for reporting; the Sponsor-Investigator will report the adverse event to the IRB as soon as possible, but in no event later than 10 calendar days, after the determination was made.

7.4 Data Safety and Monitoring Board (DSMB)

We will create an institutional internal independent DSMB committee to review the safety and toxicity data that is submitted by the study statistician every 12 months. The definitions of adverse events are described in section 7.3. Specifically we will collect expected and unexpected adverse events in Adverse Events Recording Form

(Appendix 8). The DSMB will review the frequency, grade and relationship to the study agent in the treatment group compared with the placebo group.

8. BIOSPECIMENS, BIOMARKER, AND SPECIAL STUDIES

8.1 Human Biospecimen Collection

8.1.1 Endobronchial Biopsies

Endobronchial biopsy samples will be collected during bronchoscopies. The procedure will be carried out by Dr. Wilson (Co-PI) using Narrow Band Imaging (NBI) white-light bronchoscopy. Up to 3 biopsies will be taken from each of six predetermined sites: upper lobe orifices and superior segment orifices (left and right), carina between left upper lobe division and lingular orifices and right middle lobe orifice. In addition, biopsies will be taken from any sites that appear suspicious. On the post-treatment bronchoscopy, sites that had a mild or greater grade of dysplasia at baseline will be re-biopsied.

Of the 3 tissue blocks per biopsy site. All biopsy samples will be put in an ice-cooled box and sent to Dr. Dacic (Co-I) for processing. Bronchial biopsies will be fixed in 10% neutral-buffered formalin for 6 to 24 hours, embedded in paraffin and routinely processed in the CLIA/CAP certified histology laboratory at the Department of Anatomic Pathology. One section per tissue block will be processed for routine hematoxylin-eosin (H&E) staining for histological evaluation and 10 unstained slides will be cut for immunoperoxidase studies.

8.1.2 Endobronchial Brushing

Endobronchial brushing samples will be collected from study participants during their bronchoscopy procedure using an endoscopic cytobrush (Cellebrity Endoscopic Cytobrush; Boston Scientific, Boston, MA) to collect normal appearing bronchial epithelial cells by rubbing against the bronchial wall. The bronchial brushings will be targeted to normal appearing epithelial tissue of the right main-stem bronchus and avoid sampling tumor tissue or dysplastic cells. If any abnormalities are observed on the right bronchus, the bronchial brushing samples will be collected from the left main-stem bronchus. Up to 4 bronchial brushing samples will be collected from each patient pre and post treatment, respectively. All brushing samples will be stored in an RNA preservative (RNAprotect; Qiagen) and sent to the test laboratory.

8.1.3 Bronchoalveolar Lavage

After the completion of bronchial biopsies and brushing, the bronchoscope will be wedged in the right middle lobe or lingular orifice. Up to 150 ml sterile saline will be instilled, retrieved by gentle suction. Percent recovery will be recorded. Recovered fluid will be passed through a micron sterile nylon filter (Becton Dickinson, New Jersey) to remove mucus and particulates, and pooled, and centrifuged. The BAL fluid will be harvested, aliquoted, and stored at -80^{o}C for analysis later.

8.1.4 Nasal Epithelia

Nasal brushings will be collected during clinical visits at month 0 3, 6, 9 and 12 after randomization. A nasal speculum (Bionox, Toledo, OH) then spread the nare while a standard cytology brush is inserted underneath the inferior nasal turbinate. The brush is rotated in place for 3 seconds, removed, and immediately placed in 1 ml RNA Later (Qiagen). Brushings will be obtained from the right and left inferior turbinates as previously described (73).

8.1.5 Blood

Besides a blood sample for eligibility screening or toxicity testing (10 ml), an additional blood sample (70 ml) will be collected from study participants at months 0, 3, 6, 9, and 12 after randomization. Different blood components (serum, plasma, buffy coat, RNA) in blood samples will be separated and properly stored.

8.1.6 Urine

Spot urine samples will be collected at initial screening visit (for pregnancy test for women younger than 60 years). Similarly, sport urine samples will be collected as at months 0, 3, 6, 9, and 12 after randomization. Aliquots of collected urine samples will be stored at –80^{o}C. The urine samples will be used for quantification of total ITC (dietary intake of cruciferous vegetables) and SF-NAC (for monitoring intake of SF tablets).

8.1.7 Creation of a Biospecimen Repository

Blood and urine are collected for future research that may include genetic analyses in the study. The Tissue Distribution Committee of the SF trial study is responsible for authorizing the distribution of stored DNA, serum, and urine and the determination of appropriateness of the proposed research which may include genetic analyses. This committee will review requests for the use of stored DNA, serum and urine, and provide samples to investigators if the project is scientifically and ethically acceptable. Patient samples will be provided to investigators with only the study identification number so that investigators will not know the patient's identity; thus enhancing patient confidentiality. Inadvertent discovery of non-parentage is not possible as we are only planning to collect blood from participants and not their family members. Examples of the types of genetic testing include polymorphisms in metabolizing enzymes such as GST genes. None of these genetic "susceptibility" factors would be sufficient to cause lung cancer, but they may interact with environmental exposures (such as tobacco smoke). Since this genetic testing will be performed at an unspecified time in the future, it is not possible to say that we will provide patients with any information regarding their genetic background. In the unlikely scenario that we do discover a significant gene that is clinically meaningful, we would make every attempt to contact patients with this information.

8.1.8 Identifiers

The participant's unique study ID number will be the only identifier on every specimen. Specimens will be stored in a locked -80°C freezer in the research laboratory at the UPMC Hillman Cancer Center.

8.2 Biomarkers

8.2.1 Histology (atypia)

It has long been established that abnormal bronchial histology (atypia) is associated with cigarette smoking and increased risk for lung cancer. In a follow-up study of a high-risk cohort of subjects with endoscopic biopsies, persistence of bronchial dysplasia was associated significantly with a 7-fold increased risk of developing NSCLC (adjusted hazard ratio = 7.8, 95% CI 1.6-39.4) (18). All bronchial biopsies

for histopathological evaluation will be formalin fixed, paraffin embedded, and H&E stained for subsequent morphologic evaluation and classification. Biopsies will be classified into 1 of 8 categories as defined by the World Health Organization (WHO) classification (74) and assigned a score according to the following system: 1= normal; 2= reserve cell hyperplasia; 3 = squamous metaplasia; 4 = mild dysplasia; 5 = moderate dysplasia; 6 = severe dysplasia; 7 = carcinoma in situ; and 8 = invasive carcinoma. Subjects with baseline score 7 or 8 are ineligible for the proposed study. All biopsies will be graded by the study pathologist (Dr. Dacic) in a blinded fashion as to treatment group.

Endobronchial histology scores will be first summarized within each bronchoscopy by using 3 separate endpoint measures pre and post treatment respectively: (1) The first endpoint measure is dysplasia index (DI), that solely examines changes in dysplasia pre and post-treatment. The number of biopsies with dysplasia (with WHO histology classification score: 4-6) are divided by the total number of biopsies and multiplied by 100. A positive change of 10% or greater indicates progression while a negative change of 10% or greater is regarded as a positive response. Scores in-between are coded as stable. (2) The second endpoint measure is the mean histological scores. It is calculated by adding the scores from all the biopsy sites and determining the mean value pre and post-treatment. A positive change in the score by 1 or more points is defined as progressive disease, a negative change in the score by 1 point or more points is defined as a partial or complete response and scores in-between are defined as stable disease. (3) The third model is called the "worst score". It identifies the worst histological grade pre- and post- treatment. A positive change in the score by 1 or more points is defined as progressive disease, a negative change in the score by 1 point or more points is defined as a partial or complete response and scores in-between are defined as stable disease. The final response determination for a patient requires the same response determination in 2 of the 3 measures. For the rare patient with a different response for each category (progression, stable and response) the patient will be deemed stable. These summary scores will be created by the study statistician with the input of all investigators, in particular Dr. Dacic.

8.2.2 Cell proliferation (Ki-67 labeling index)

A common endpoint of pathways affected by sulforaphane is cell proliferation. Ki-67 labeling is a validated indicator of proliferative index and can be readily detected in bronchial biopsy material (75-77). Ki-67 labeling index (Ki-67 LI) is positively associated with increased grade of bronchial dysplasia; a mean of 4% in normal and hyperplastic samples increased to 38% in biopsies with moderate or severe dysplasia (Table 4). When a serial set of matched biopsies were examined for change over time there was correlation between histological change and Ki-67 labeling scores. Ki-67 thus is an easily performed proliferative index that will be a primary endpoint in this study.

Table 4. Histology and Biomarkers in Bronchial Epithelium of High Risk Subjects

Study group	Histology	Ki-67 (mean %)
All subjects	Grade 1-2 (N=14 biopsies)	4%
	Grade 3-4 (N=16 biopsies)	20%

	Grade 5-6 (N=39 biopsies)	38%
Serial Biopsies:		
No change (N=7)	Mean grade change 0	-17%
Progression (N=10)	Mean grade change +2	+17%
Improvement (N=4)	Mean grade change –2.5	-21%

8.2.3 Apoptosis (TUNEL and Caspase-3)

Like the proliferative index, the apoptotic index will provide a measure of a final common pathway. Dietary SF significantly induced markers of apoptosis in lung tissue of mice after treated with tobacco carcinogens. Compared with control animals, mice given SF showed a statistically significant 6-fold increase (from 4.7±1.9 to 29.3±4.7%) in activated caspase-3, and a 2-fold increase (from 12.7±3.0 to 29.4±5.8%) in TUNEL (terminal transferase dUTP nick end labeling) of lung cells. Activated caspase-3 and TUNEL are representative markers for apoptosis (14). Thus we will perform TUNEL and caspas-3 assays on sections of bronchial mucosa from histologically normal and abnormal sites pre and post SF treatment.

8.2.4 Gene expression markers

Gene expression in the airway epithelium, which can be sampled by bronchoscopic and nasal brushings, represents an intermediate biological endpoint between underlying genetic or epigenetic alterations driving carcinogenesis and the clinical diagnosis of lung cancer. Dr. Spira (Co-I) and colleagues (19) recently validated that a 23-gene expression marker in epithelial cells collected from the normal-appearing main-stem bronchus significantly improved the diagnostic performance of bronchoscopy for detection of lung cancer. The combination of the 23-gene expression marker plus bronchoscopy had a sensitivity of 96% (95% CI, 93% to 98%) in the first cohort of patients and 98% (95% CI, 96% to 99%) in the second cohort of patients, independent of lesion size and location. In patients with a nondiagnostic bronchoscopic examination, the gene-expression classifier accurately identified cancer in 89%. The 23-gene expression marker is the first genomic classifier to improve the diagnostic performance of bronchoscopy for the detection of lung cancer. In the proposed study, we will evaluate if the SF treatment alters the gene expression of these 23 genes in the bronchial epithelium, and decreases the classifier score towards that seen with lung benign disease (78).

Using mRNA sequencing (mRNA-Seq), we profiled cytologically normal airway epithelial cells collected during auto-fluorescence bronchoscopy from high-risk smokers with (n=50) and without (n=25) bronchial premalignant lesions (PMLs). Using surrogate variable analysis, we identified 280 genes significantly differentially expressed between subjects with and without PMLs at false discovery rate (FDR)<0.002. The 280-gene signature has a significant concordant relationship to gene expression changes identified in PMLs adjacent to non-small cell lung cancer (NSCLC), in NSCLC tumors, and in the field of individuals with lung cancer (FDR<0.05). These data suggest that the change in certain gene expressions in bronchial epithelium takes place at premalignant stage. In the proposed study, we will

explore if the SF treatment alters the gene expression profile of these 280 PMLs-related genes in bronchial epithelium of the study participants.

The unified airway field of injury concept suggests that cigarette smoking renders injury throughout the entire respiratory tract. Studies have shown that tobacco-related gene expression alterations in the extra-thoracic epithelium of the nose and mouth similarly reflect the host response to cigarette smoke in the distal airway (79). Both nasal and bronchial epithelial cells demonstrate similar patterns of gene expression changes in response to cigarette smoking (73), suggesting that the nasal epithelium may be a surrogate biospecimen for monitoring the individual response to smoking and potentially the risk for lung cancer. More recently, we have demonstrated that the cancer-specific gene-expression changes in the bronchial epithelium are enriched among gene-expression alterations observed in the nasal epithelium from approximately 500 smokers with matched nasal and bronchial samples. These data suggest that genomic profiling of these relatively accessible airway cells may be an intermediate endpoint of chemoprevention for lung cancer. In the proposed study, we will explore whether SF-induced gene expression changes in bronchial epithelium mirror those in the nasal epithelium.

8.3 End of Treatment Evaluation

8.3.1 Histological Response

Histology on bronchial biopsies pre-treatment and post-treatment will be compared. All biopsies will be graded according to the WHO classification for bronchial epithelium (74):

WHO Classification	**Grade**
Normal	1.0
Reserve Cell Hyperplasia	2.0
Metaplasia	3.0
Mild Dysplasia	4.0
Moderate Dysplasia	5.0
Severe Dysplasia	6.0
Carcinoma in Situ	7.0
Carcinoma	8.0

Response will then be determined by three scoring methods:

a) The worst histology grade per patient pre and post treatment.

Worse = ≥ 1 point higher post treatment
Better = ≥ 1 point lower post treatment
Same = same point score pre and post treatment

b) Average histology (all biopsies scores/number of biopsies) pre and post treatment

Worse = ≥ 1 point higher post treatment
Better = ≥ 1 point lower post treatment
Same = same point score pre and post treatment

c) Dysplasia index (number of biopsies with dysplasia/total number of biopsies x 100) pre and post treatment.

Worse = $\geq$ 10% higher post treatment
Better = $\geq$ 10% lower post treatment
Same = < 10% difference post treatment

The final response determination will require the same response parameter (worse, better or the same) for 2 of the 3 methods.

8.3.2 Cellular Biomarker Response

A secondary endpoint is to determine if SF can modulate a proliferative intermediate marker Ki-67. Based on prior studies, a response will be considered as a decrease of > 10 in the labeling index, progression will be an increase of > 10 in the labeling index and no change will signify an increase or decrease of $\leq$ 10.

We will also evaluate other biomarkers including apoptotic biomarkers TUNEL and caspase-3.

8.3.3 Gene Expression Response

The exact mechanisms for the observed SF-mediated 44 hemoprotection remain to be elucidated. The upregulation of NQO1 transcript suggests the activation of Nfr2 pathway (59). The alteration of lung cancer associated gene expression in BAL is an indication of SF's impact on lung cancer risk in the study population.

8.3.4 Safety and Toxicity Assessment

The safety and toxicity will be assessed by the study physician at the research clinic once every three months. We plan to closely monitor all study patients for potential SF-related adverse events. This includes both expected (based on previous clinical experience with oral SF) and unexpected events. The expected adverse events (AEs) are gastrointestinal including taste alternation (bad taste), dry mouth, belching, nausea, vomiting, and diarrhea. The frequencies of AEs were low and most were at moderate by grade (see Table 3).

9. STATISTICAL AND SAMPLE SIZE CONSIDERATIONS

This is a randomized, double blind, placebo-controlled study to evaluate the efficacy of SF as a chemopreventive agent. Eligible subjects are former smokers with ≥30 pack-years smoking history who quit smoking <15 years, and are considered at high risk for developing lung cancer. The proposed duration of therapy will be 12 months for each subject. The primary objective of this trial is to assess whether oral SF supplementation elicits an increased histologic response when compared to placebo in this population over a 12-month treatment period. The planned sample size is 72 subjects (36 per treatment arm). A block randomization scheme will be used to ensure that both treatment and placebo group have the same proportion of men and women, respectively. The randomization will be done at the UPMC Hillman Cancer Center.

9.1 Statistical Analysis for histological responses

Endobronchial histology scores will be first summarized within each bronchoscopy by using 3 separate endpoint measures pre and post treatment respectively: 1) dysplasia index (DI, number of biopsies with dysplasia/total number of biopsies x 100), 2) average of all histological scores (all biopsies scores/number of biopsies), and 3) worst biopsy score. The 3 summary end points will be analyzed within 4 different biopsy site groupings: all biopsies, biopsies from the 6 standard endobronchial sites only, site-matched biopsies from both bronchoscopies, and site-matched biopsies where the baseline biopsy is non-normal. For each patient, the mean of each of endpoints separately for each of biopsy site groupings pre and post treatment are then calculated. The primary endpoint is the dysplasia index (DI), which has a binomial distribution with at least 6 "trials" (i.e., 6 predetermined biopsy sites), and will be analyzed by generalized estimating equations (GEE) that constrains average baseline DI to be the same for both treatment and placebo groups, and tests whether average follow-up DI differs between the SF treatment and placebo groups. All other endpoints and biopsy site groupings are secondary endpoints. The primary analysis for the average and worst dysplasia scores will be a linear regression that assesses the difference in post-treatment scores by the two treatment groups, controlling for the same scores at pre-treatment. This analysis of covariance (ANCOVA) approach has optimal efficiency in a study design to assess the pre- and post-treatment measures with high within-subject correlation due to repeated measures (80). No interim analyses for futility or superiority will be performed, other than ongoing safety monitoring.

Sample size consideration. In the iloprost trial, 57 former smokers were randomized to receive oral iloprost or placebo for 6 months. Treatment effects (adjusted for baseline levels) were statistically significant ($P \leq 0.01$) for all 3 endpoints (dysplasia index, average dysplasia score, worst dysplasia score). Anticipating a 20% dropout rate over 12 months, we will randomize at least 72 study subjects to ensure that 60 subjects complete our SF study. Assuming an average baseline dysplasia index of 22% and post-therapy 11% for subjects receiving SF, a simulation with 10,000 trials with a sample size of 60 subjects has yielded an 80% statistical power to detect a statistically significant (two-sided $p<0.05$) treatment effect, using generalized linear models (binomial family, 6 biopsies) after controlling for baseline levels of DI. Calculations are similar for other endpoints, and actual power should be greater than the DI measure because they are continuous measures and have observed within-subject correlation (i.e., repeated measures).

9.2 Statistical Analysis for Cellular Proliferation/Apoptosis Markers

Ki-67, caspase-3 and TUNEL will be quantified as % positive cells in two slides of each tissue block. First we calculate the average values of each of the 3 IHC markers over 6 tissue blocks within each bronchoscopy per patient separately for pre- and post-treatment. As described above for bronchial dysplasia measures, the primary analysis for these continuous measures will be a linear regression predicting post-treatment score by treatment group, controlling for pre-treatment score. We hypothesize that SF treated subjects will exhibit lower expression of Ki-67 and higher expression of caspase-3 and TUNEL relative to subjects in the placebo arm.

Sample Size Consideration. Using estimates of the mean and standard deviation (12.7±8.2) of Ki-67 LI in the para-basal layer of bronchial epithelia of smokers (15) and the logit function, we back calculated plausible estimates of the mean (2.3676) and

variance (0.3480) of log-transformed baseline Ki-67 LI (81). Assuming these log-transformed Ki-67 LI values are normally distributed, we simulated 10,000 sets of data (n=60 each) and found an 84% statistical power with a two-sided $P<0.05$ to detect a treatment effect size of 37% for change in Ki-67 LI after 12 months of SF treatment (i.e., median Ki-67 LI of 10.7% at baseline and 6.7% at follow-up on SF). This detectable effect size is smaller than 61% reduction in cell proliferation marker in the SF treatment group (14). Furthermore, a recent randomized, double-blind, placebo-controlled phase 2 clinical trial involving 58 women found that oral intake of SF glucosinolate for 2-8 weeks resulted in a statistically significant reduction of mean Ki-67 LI (P = 0.01) (16).

9.3 Statistical Analysis for Gene Expression

A summary gene expression score will be derived from previously determined gene expression features of 23 genes (19,82): score = $e^y/(1+e^y)$, where y = $b_0 + \sum b_j * x_j$, where b_0 is the intercept, b_j is the coefficient, and x_j is the feature of the gene j expression [see details in (82)]. We will calculate the gene expression score (0~1) for each subject pre and post treatment separately. A linear mixed effect model will be used to assess the effect of SF on post-treatment gene expression scores, similar as described in Section 4.2.1. A decreased gene expression score post-treatment towards those seen in bronchial benign disease is indicative of the chemopreventive effect of SF on the lung cancer-gene expression.

We will use the similar approach to calculate a summary gene expression score of the 280-gene signature in bronchial premalignant lesions (PMLs) in identified our preliminary study. Similarly a linear mixed effect model will be used to assess the post-treatment gene expression scores. If SF causes a significant change of the score towards the regression of PMLs based on histological dysplasia index score, the gene-level expression changes will be examined to estimate the SF-treatment effects on individual gene or a cluster of genes.

To assess the similarity and difference in the gene expressions between the bronchial airway and nasal epithelia, we will use our similar methodologies described previously (73). For each gene identified in the analysis above, we will use a linear mixed effects model that includes site (bronchus or nose) and treatment (SF or placebo) as fixed effect. The regression model is given: $G_{ij} \sim \mu + \beta_s \bullet X_s + \beta_t \bullet X_t + \beta_{st} \bullet X_s \bullet X_t + \varepsilon_{ij}$, where G_{ij} is the $\log_2$ expression value for gene i in patient j; the parameter μ is the overall mean of $\log_2$ fold change in gene expression, β_s and β_t for the respective site- and treatment-specific effect, and β_{st} for the interaction between site and treatment; and X_s for site (1=nose, 0=bronchus) and X_t for treatment (1=SF, 0=placebo).

Sample Size Consideration. The Affymetrix Human Gene 1.0 ST Array contains ~36,000 gene transcripts. The proposed study with 30 subjects per group will have a 85% power to detect a 2-fold gene expression difference pre and post treatment ($\log_2 |d|=1.0$, $\sigma_d = 0.7$) for 23 identified cancer-related genes at a false discovery rate (FDR)<0.05 using the method of Liu and Hwang (83), assuming 99.9% non-differentially expressed genes ($\pi_0 = 0.999$). Similarly the study will have a 96% power for 280 PMLs-related genes (i.e., $\pi_0 = 0.990$), and a 99% power for 5% differentially expressed genes (i.e., $\pi_0 = 0.950$) in exploratory analysis.

9.4 Statistical Analysis for Adverse Events and Toxicity Measures

The frequencies of adverse events (AEs) and toxicity measures (e.g., CBC, liver function test, body weight) will be compared between the SF treatment and placebo arm. The analysis will be conducted for total AEs and specific AEs, expected AEs and unexpected AEs, AEs by grade and attribution grouping, respectively, at each clinical visit as well as the summed total over 4 time points of the entire treatment period. We will repeat the same analysis on a patient level. Chi-square test will be used for comparing the frequencies between the two treatment groups.

Sample Size Consideration. As described in Table 3 above, the frequencies of AEs reported were low in previous studies. Based on the frequencies repored by Atwell et al, the frequency of total AE in placebo was 33% (9 out of 27 participants), the proposed study with a total of 72 subjects will have an 80% statistical power to detect a doubled frequency (67%) of total AEs in the SF-treatment group. The proposed study can detect a less excess AE rate between the two treatment groups if all AEs over different time points are combined or all AEs are counted independently.

10. GENDER AND MINORITY INCLUSION

The University of Pittsburgh and UPMC are committed to providing access to cancer clinical trials, both therapeutic and non-therapeutic, for all citizens of their state and region, including their under-served populations. Every effort is made to provide access to clinical trials for all individuals and special emphasis is placed on retaining those who are enrolled.

At enrollment, PluSS participants had a mean age of 60 years. Among the participants, 49% were women; 93% whites, 5.5% blacks, and 1.7% other race/ethnicity groups; 60% current smokers and 40% former smokers with a median 47 pack-years of smoking history (61). The PluSS participants resembled the national representative sample of current and former smokers who are at high risk for lung cancer. For example, the National Lung Screening Trial (NLST) enrolled more than 53,000 persons at high risk for lung cancer at 33 medical centers across the US. The mean age was 61 years. Forty-one % were women; 91% whites, 4% blacks, and 5% other race/ethnicity groups; 48% current smokers and 52% former smokers who quit smoking within the previous 15 years; a median 48 pack-years of smoking history (62).

11. ETHICAL AND REGULATORY CONSIDERATIONS

The following must be observed to comply with Food and Drug Administration (FDA) regulations for the conduct and monitoring of clinical investigations; they also represent sound research practice:

11.1 Informed Consent

Patient consent is solicited in the clinic by Co-Principal Investigators or the designated trained study staff. Obtaining subject informed consent will be done in a setting that facilitates information transfer that must minimally include, unhurried time between consent and the initiation of study procedures and a quiet setting. The subject will be provided the written Consent Form (see a copy at the end of the Clinical Protocol) with time to read it. The subject will be asked questions explaining

the study in their own words to insure understanding. A copy of the signed consent will be provided to subject.

The principles of informed consent are described by Federal Regulatory Guidelines (Federal Register Vol. 46, No. 17, January 27, 1981, part 50). They must be followed to comply with FDA regulations for the conduct and monitoring of clinical investigations. In seeking informed consent, the following information shall be provided in a language understandable to the subject.

11.2 Basic Elements of Informed Consent

The following are the basic elements of informed consent which should be provided to each subject:

a) A statement that the study involves research, an explanation of the purposes of the research, and the expected duration of the subject's participation, a description of the procedures to be followed, and the identification of any procedures which are experimental.

b) A description of any reasonably foreseeable risks or discomforts to the subject.

c) A description of any benefits to the subject or to others which may reasonably be expected from research.

d) A disclosure of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the subject.

e) A statement describing the extent, if any, to which confidentiality of records identifying the subject will be maintained and that notes the possibility that the FDA may inspect the records.

f) For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether any medical treatments are available if injury occurs and, if so, what they consist of, or where further information may be obtained.

g) An explanation of whom to contact for answers for pertinent questions about the research and research subjects' rights, and whom to contact in the event of a research-related injury to the subject.

h) A statement that participation is voluntary, that refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and that the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

11.3 Additional Elements of Informed Consent

When appropriate, one or more of the following elements of information shall also be provided to each subject:

a) A statement that the particular treatment or procedure may involve risks to the subject which are currently foreseeable.

b) Anticipated circumstances under which the subject's participation may be terminated by the investigator without regard to the subject's consent.

c) Any additional costs to the subject that may result from participation in the research

d) The consequences of the subject's decision to withdraw from the research and procedures for orderly termination of participation by subject.

e) A statement that significant new findings developed during the course of the research which may relate to the subject's willingness to continue participation will be provided to the subject.

f) The approximate number of subjects involved in the study.

A subject must give his/her written consent to participate in the study. This consent must be witnessed and dated and retained by the investigator as part of the study records.

If Experimental Subject's Bill of Rights is applicable in your state, this form must also be prepared and signed by each subject and retained as part of the required study records.

A copy of the proposed consent form must be submitted to the Institutional Review Board together with the protocol for approval. Each subject's signed informed consent form must be kept of file by the investigator for FDA inspection at any time.

11.4 Institutional Review

This study must be approved by an appropriate institutional review committee as defined by Federal Regulatory Guidelines (Ref. Federal register Vol. 46, No. 17, January 27, 1981, part 56).

The protocol and informed consent form for this study must be approved in writing by the appropriate Institutional Review Board (IRB). The IRB must be from an institution which has a valid Multiple Project Assurance, Single Project Assurance, and Cooperative Oncology Group Assurance on file with the Office for Protection from Research Risks, National Institutes of Health. The institution must be in compliance with regulations of the FDA and the Department of Health and Human Services.

Significant changes to the protocol, as well as a change of principal investigators, must also be approved by the IRB and documentation of the approval provided to the study monitor. Records of the IRB review and approval of all documents pertaining to this study must be kept on file by the investigator and are subject to FDA inspection at any time during this study. Periodic status reports must be submitted to the IRB at least yearly, as well as notification of completion of the study and a final report within 3 months of study completion or termination. The investigator must maintain an accurate and complete record of all submissions made to the IRB including a list of all reports and documents submitted.

11.5 Drug Accountability

For each drug supplied for a study, an accountability ledger containing current and accurate inventory records covering receipt, dispensing, and the return of study drug supplies must be maintained. Drug supplies must be kept in a secure, limited access storage area under the recommended storage conditions. During the course of the study, the following information must be noted on the accountability ledger; the identification code of the subject to whom drug is dispensed, the date(s) and quantity of drug dispensed to the subject, and the date(s) and quantity of drug returned by the subject; subjects should return empty containers to the investigator, with the return noted on the ledger. These Accountability Forms must be readily available for inspection and are open to FDA inspection at any time.

12. STUDY DATA MANAGEMENT

All case report forms (CRFs) and specimens for the UPMC Hillman Cancer Center (Hillman) and the multi-institutional centers will be kept and reviewed at Hillman. Patient confidentiality is maintained on study CRFs by use of assigned Study ID#. Records for this study will be maintained in locked cabinets in the study coordinator office in the secured file room that can be accessed only by the investigators and staff on the research team. Tissue, blood and urine specimens are given a specimen ID number that is linked to the study ID#. Patient names do not appear in the pathology database. Specimens are provided to investigators using specimen ID# as the only identification on the sample. The Principal Investigator oversees access to the study database with only authorized investigators receiving access. Data sets provided to the investigators do not contain patients names with subjects being identified by study ID# only. Access to the main database where patient identification is available is secured using password access from identified net locations only. Delinking of subject ID with specimen information occurs prior to the sharing of specimens with other institutions. At the end of the study, completed records are locked in the Clinical Investigations storeroom.

13. FUNDING

This study has been funded by NIH from 6/14/2017 through 5/31/2022 (R01 CA213123, PI Jian-Min Yuan).

14. REFERENCES

1. Hu R, Hebbar V, Kim BR, Chen C, Winnik B, Buckley B, Soteropoulos P, Tolias P, Hart RP, Kong AN. In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. *J Pharmacol Exp Ther* **310**:263-271, 2004.

2. Forman D, Ferlay J. The global and regional burden of cancer. In: Stewart BW, Wild CP, editors. World Cancer Report 2014. Lyon, France: International Agency for Research on Cancer; 2014. p. 16-53.

3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* **65**:5-29, 2015.

4. U.S. Department of Health and Human Services. The Health Consequences of Smoking-50 Years of Progress: A Report of the Surgeon General. Atlanta (GA): U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; 2014.

5. Lavinghouze SR, Malarcher A, Jama A, Neff L, Debrot K, Whalen L. Trends in Quit Attempts Among Adult Cigarette Smokers - United States, 2001-2013. *MMWR Morb Mortal Wkly Rep* **64**:1129-1135, 2015.

6. Jha P, Ramasundarahettige C, Landsman V, Rostron B, Thun M, Anderson RN, McAfee T, Peto R. 21st-century hazards of smoking and benefits of cessation in the United States. *N Engl J Med* **368**:341-350, 2013.

7. Tong L, Spitz MR, Fueger JJ, Amos CA. Lung carcinoma in former smokers. *Cancer* **78**:1004-1010, 1996.

8. American Cancer Society. Cancer Facts & Figures 2013. Atlanta; 2013.

9. Chung FL, Jiao D, Getahun SM, Yu MC. A urinary biomarker for uptake of dietary isothiocyanates in humans. *Cancer Epidemiol Biomarkers Prev* **7**:103-108, 1998.

10. Jiao D, Yu MC, Hankin JH, Low SH, Chung FL. Total isothiocyanate contents in cooked vegetables frequently consumed in Singapore. *J Agric Food Chem* **46**:1055-1058, 1998.

11. London SJ, Yuan J-M, Chung FL, Gao YT, Coetzee GA, Ross RK, Yu MC. Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China. *Lancet* **356**:724-729, 2000.

12. Lam TK, Gallicchio L, Lindsley K, Shiels M, Hammond E, Tao XG, Chen L, Robinson KA, Caulfield LE, Herman JG, Guallar E, Alberg AJ. Cruciferous vegetable consumption and lung cancer risk: a systematic review. *Cancer Epidemiol Biomarkers Prev* **18**:184-195, 2009.

13. Hecht SS, Trushin N, Rigotty J, Carmella SG, Borukhova A, Akerkar S, Rivenson A. Complete inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced rat lung

tumorigenesis and favorable modification of biomarkers by phenethyl isothiocyanate. *Cancer Epidemiol Biomarkers Prev* **5**:645-652, 1996.

14. Conaway CC, Wang CX, Pittman B, Yang YM, Schwartz JE, Tian D, McIntee EJ, Hecht SS, Chung FL. Phenethyl isothiocyanate and sulforaphane and their N-acetylcysteine conjugates inhibit malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice. *Cancer Res* **65**:8548-8557, 2005.

15. Lee JJ, Liu D, Lee JS, Kurie JM, Khuri FR, Ibarguen H, Morice RC, Walsh G, Ro JY, Broxson A, Hong WK, Hittelman WN. Long-term impact of smoking on lung epithelial proliferation in current and former smokers. *J Natl Cancer Inst* **93**:1081-1088, 2001.

16. Atwell LL, Zhang Z, Mori M, Farris PE, Vetto JT, Naik AM, Oh KY, Thuillier P, Ho E, Shannon J. Sulforaphane Bioavailability and Chemopreventive Activity in Women Scheduled for Breast Biopsy. *Cancer Prev Res (Phila)* **8**:1184-1191, 2015.

17. Egner PA, Chen JG, Zarth AT, Ng DK, Wang JB, Kensler KH, Jacobson LP, Munoz A, Johnson JL, Groopman JD, Fahey JW, Talalay P, Zhu J, Chen TY, Qian GS, Carmella SG, Hecht SS, Kensler TW. Rapid and sustainable detoxication of airborne pollutants by broccoli sprout beverage: results of a randomized clinical trial in China. *Cancer Prev Res (Phila)* **7**:813-823, 2014.

18. Merrick DT, Gao D, Miller YE, Keith RL, Baron AE, Feser W, Kennedy TC, Blatchford PJ, Braudrick S, Hirsch FR, Heasley L, Paul A. Bunn PAJ, Franklin WA. Persistence of bronchial dysplasia is associated with development of invasive squamous cell carcinoma. *Cancer Prev Res (Phila Pa)* **9**:(in press), 2016.

19. Silvestri GA, Vachani A, Whitney D, Elashoff M, Porta Smith K, Ferguson JS, Parsons E, Mitra N, Brody J, Lenburg ME, Spira A, Team AS. A Bronchial Genomic Classifier for the Diagnostic Evaluation of Lung Cancer. *N Engl J Med* **373**:243-251, 2015.

20. Albanes D, Heinonen OP, Taylor PR, Virtamo J, Edwards BK, Rautalahti M, Hartman AM, Palmgren J, Freedman LS, Haapakoski J, Barrett MJ, Pietinen P, Malila N, Tala E, Liippo K, Salomaa ER, Tangrea JA, Teppo L, Askin FB, Taskinen E, Erozan Y, Greenwald P, Huttunen JK. Alpha-Tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. *J Natl Cancer Inst* **88**:1560-1570, 1996.

21. Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med* **334**:1150-1155, 1996.

22. Keith RL, Blatchford PJ, Kittelson J, Minna JD, Kelly K, Massion PP, Franklin WA, Mao J, Wilson DO, Merrick DT, Hirsch FR, Kennedy TC, Bunn PA, Jr., Geraci MW, Miller YE. Oral iloprost improves endobronchial dysplasia in former smokers. *Cancer Prev Res (Phila)* **4**:793-802, 2011.

23. Mao JT, Roth MD, Fishbein MC, Aberle DR, Zhang ZF, Rao JY, Tashkin DP, Goodglick L, Holmes EC, Cameron RB, Dubinett SM, Elashoff R, Szabo E, Elashoff D. Lung cancer chemoprevention with celecoxib in former smokers. *Cancer Prev Res (Phila)* **4**:984-993, 2011.

24. Mao JT, Fishbein MC, Adams B, Roth MD, Goodglick L, Hong L, Burdick M, Strieter ER, Holmes C, Tashkin DP, Dubinett SM. Celecoxib decreases Ki-67 proliferative index in active smokers. *Clin Cancer Res* **12**:314-320, 2006.

25. Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* **56**:5-51, 2001.

26. Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer Epidemiol Biomarkers Prev* **7**:1091-1100, 1998.

27. Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. *Cancer Epidemiol Biomarkers Prev* **10**:501-508, 2001.

28. Brooks JD, Paton VG, Vidanes G. Potent induction of phase 2 enzymes in human prostate cells by sulforaphane. *Cancer Epidemiol Biomarkers Prev* **10**:949-954, 2001.

29. Cornblatt BS, Ye L, Dinkova-Kostova AT, Erb M, Fahey JW, Singh NK, Chen MS, Stierer T, Garrett-Mayer E, Argani P, Davidson NE, Talalay P, Kensler TW, Visvanathan K. Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. *Carcinogenesis* **28**:1485-1490, 2007.

30. Fahey JW, Talalay P, Kensler TW. Notes from the field: "green" chemoprevention as frugal medicine. *Cancer Prev Res (Phila)* **5**:179-188, 2012.

31. Shapiro TA, Fahey JW, Dinkova-Kostova AT, Holtzclaw WD, Stephenson KK, Wade KL, Ye L, Talalay P. Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: a clinical phase I study. *Nutr Cancer* **55**:53-62, 2006.

32. Kensler TW, Chen JG, Egner PA, Fahey JW, Jacobson LP, Stephenson KK, Ye L, Coady JL, Wang JB, Wu Y, Sun Y, Zhang QN, Zhang BC, Zhu YR, Qian GS, Carmella SG, Hecht SS, Benning L, Gange SJ, Groopman JD, Talalay P. Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. *Cancer Epidemiol Biomarkers Prev* **14**:2605-2613, 2005.

33. Zhang Y, Talalay P. Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes. *Cancer Res* **58**:4632-4639, 1998.

34. Zhang Y. Molecular mechanism of rapid cellular accumulation of anticarcinogenic isothiocyanates. *Carcinogenesis* **22**:425-431, 2001.

35. Zhang Y. Role of glutathione in the accumulation of anticarcinogenic isothiocyanates and their glutathione conjugates by murine hepatoma cells. *Carcinogenesis* **21**:1175-1182, 2000.

36. Zhang Y, Callaway EC. High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate. *Biochem J* **364**:301-307, 2002.

37. Keck AS, Staack R, Jeffery EH. The cruciferous nitrile crambene has bioactivity similar to sulforaphane when administered to Fischer 344 rats but is far less potent in cell culture. *Nutr Cancer* **42**:233-240, 2002.

38. Gerhauser C, You M, Liu J, Moriarty RM, Hawthorne M, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive potential of sulforamate, a novel analogue of sulforaphane that induces phase 2 drug-metabolizing enzymes. *Cancer Res* **57**:272-278, 1997.

39. Yoxall V, Kentish P, Coldham N, Kuhnert N, Sauer MJ, Ioannides C. Modulation of hepatic cytochromes P450 and phase II enzymes by dietary doses of sulforaphane in rats: Implications for its chemopreventive activity. *Int J Cancer* **117**:356-362, 2005.

40. Kassahun K, Davis M, Hu P, Martin B, Baillie T. Biotransformation of the naturally occurring isothiocyanate sulforaphane in the rat: identification of phase I metabolites and glutathione conjugates. *Chem Res Toxicol* **10**:1228-1233, 1997.

41. Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E. Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. *Exp Biol Med (Maywood)* **232**:227-234, 2007.

42. Jones SB, Brooks JD. Modest induction of phase 2 enzyme activity in the F-344 rat prostate. *BMC Cancer* **6**:62, 2006.

43. Zhang Y, Munday R, Jobson HE, Munday CM, Lister C, Wilson P, Fahey JW, Mhawech-Fauceglia P. Induction of GST and NQO1 in cultured bladder cells and in the urinary bladders of rats by an extract of broccoli (Brassica oleracea italica) sprouts. *J Agric Food Chem* **54**:9370-9376, 2006.

44. Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, Talalay P. Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. *Clin Chim Acta* **316**:43-53, 2002.

45. Fahey JW, Talalay P. Antioxidant functions of sulforaphane: a potent inducer of Phase II detoxication enzymes. *Food Chem Toxicol* **37**:973-979, 1999.

46. Egner PA, Chen JG, Wang JB, Wu Y, Sun Y, Lu JH, Zhu J, Zhang YH, Chen YS, Friesen MD, Jacobson LP, Munoz A, Ng D, Qian GS, Zhu YR, Chen TY, Botting NP, Zhang Q, Fahey JW, Talalay P, Groopman JD, Kensler TW. Bioavailability of

Sulforaphane from two broccoli sprout beverages: results of a short-term, cross-over clinical trial in Qidong, China. *Cancer Prev Res (Phila Pa)* **4**:384-395, 2011.

47. Fowke JH, Fahey JW, Stephenson KK, Hebert JR. Using isothiocyanate excretion as a biological marker of Brassica vegetable consumption in epidemiological studies: evaluating the sources of variability. *Public Health Nutr* **4**:837-846, 2001.

48. Fahey JW, Holtzclaw WD, Wehage SL, Wade KL, Stephenson KK, Talalay P. Sulforaphane Bioavailability from Glucoraphanin-Rich Broccoli: Control by Active Endogenous Myrosinase. *PLoS ONE* **10**:e0140963, 2015.

49. Seow A, Shi CY, Franke AA, Hankin JH, Lee HP, Yu MC. Isoflavonoid levels in spot urine are associated with frequency of dietary soy intake in a population-based sample of middle-aged and older Chinese in Singapore. *Cancer Epidemiol Biomarkers Prev* **7**:135-140, 1998.

50. Nugon-Baudon L, Rabot S. Glucosinolates and glucosinolate derivatives: implications for protection against chemical carcinogenesis. *Nutr Res Rev* **7**:205-231, 1994.

51. Milford GFJ, Evans EJ. Factors causing variation in glucosinolates in oilseed rape. . *Outlook Agriculture* **20**:31-37, 1991.

52. Mullin WJ, Sahasrabudhe HR. An estimate of the average daily intake of glucosinolate. . *Nutr Rep Int* **18**:273-279, 1978.

53. Wattenberg LW, Hanley AB, Barany G, Sparnins VL, Lam LKT, Fenwick GR. Inhibition of carcinogenesis by some minor constituents. In: Hayashi Y, editor. Diet, Nutrition and Cancer. Tokyo: Japan Science Society Press; 1986. p. 193-203.

54. Murashima M, Watanabe S, Zhuo XG, Uehara M, Kurashige A. Phase 1 study of multiple biomarkers for metabolism and oxidative stress after one-week intake of broccoli sprouts. *Biofactors* **22**:271-275, 2004.

55. Riedl MA, Saxon A, Diaz-Sanchez D. Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway. *Clin Immunol* **130**:244-251, 2009.

56. Christiansen B, Bellostas Muguerza N, Petersen AM, Kveiborg B, Madsen CR, Thomas H, Ihlemann N, Sorensen JC, Kober L, Sorensen H, Torp-Pedersen C, Dominguez H. Ingestion of broccoli sprouts does not improve endothelial function in humans with hypertension. *PLoS ONE* **5**:e12461, 2010.

57. Bahadoran Z, Mirmiran P, Hosseinpanah F, Hedayati M, Hosseinpour-Niazi S, Azizi F. Broccoli sprouts reduce oxidative stress in type 2 diabetes: a randomized double-blind clinical trial. *Eur J Clin Nutr* **65**:972-977, 2011.

58. Alumkal JJ, Slottke R, Schwartzman J, Cherala G, Munar M, Graff JN, Beer TM, Ryan CW, Koop DR, Gibbs A, Gao L, Flamiatos JF, Tucker E, Kleinschmidt R, Mori M. A phase II study of sulforaphane-rich broccoli sprout extracts in men with recurrent prostate cancer. *Invest New Drugs* **33**:480-489, 2015.

59. Bauman JE, Zang Y, Sen M, Li C, Wang L, Egner PA, Fahey JW, Normolle DP, Grandis JR, Kensler TW, Johnson DE. Prevention of Carcinogen-Induced Oral Cancer by Sulforaphane. *Cancer Prev Res (Phila)* **9**:547-557, 2016.

60. Atwell LL, Hsu A, Wong CP, Stevens JF, Bella D, Yu TW, Pereira CB, Lohr CV, Christensen JM, Dashwood RH, Williams DE, Shannon J, Ho E. Absorption and chemopreventive targets of sulforaphane in humans following consumption of broccoli sprouts or a myrosinase-treated broccoli sprout extract. *Mol Nutr Food Res* **59**:424-433, 2015.

61. Wilson DO, Weissfeld JL, Balkan A, Schragin JG, Fuhrman CR, Fisher SN, Wilson J, Leader JK, Siegfried JM, Shapiro SD, Sciurba FC. Association of radiographic emphysema and airflow obstruction with lung cancer. *Am J Respir Crit Care Med* **178**:738-744, 2008.

62. National Lung Screening Trial Research T, Aberle DR, Adams AM, Berg CD, Clapp JD, Clingan KL, Gareen IF, Lynch DA, Marcus PM, Pinsky PF. Baseline characteristics of participants in the randomized national lung screening trial. *J Natl Cancer Inst* **102**:1771-1779, 2010.

63. Wilson DO, Weissfeld JL, Fuhrman CR, Fisher SN, Balogh P, Landreneau RJ, Luketich JD, Siegfried JM. The Pittsburgh Lung Screening Study (PLuSS): outcomes within 3 years of a first computed tomography scan. *Am J Respir Crit Care Med* **178**:956-961, 2008.

64. Styn MA, Land SR, Perkins KA, Wilson DO, Romkes M, Weissfeld JL. Smoking behavior 1 year after computed tomography screening for lung cancer: Effect of physician referral for abnormal CT findings. *Cancer Epidemiol Biomarkers Prev* **18**:3484-3489, 2009.

65. Wilson DO, Leader JK, Fuhrman CR, Reilly JJ, Sciurba FC, Weissfeld JL. Quantitative computed tomography analysis, airflow obstruction, and lung cancer in the pittsburgh lung screening study. *J Thorac Oncol* **6**:1200-1205, 2011.

66. de-Torres JP, Wilson DO, Sanchez-Salcedo P, Weissfeld JL, Berto J, Campo A, Alcaide AB, Garcia-Granero M, Celli BR, Zulueta JJ. Lung Cancer in Patients with Chronic Obstructive Pulmonary Disease. Development and Validation of the COPD Lung Cancer Screening Score. *Am J Respir Crit Care Med* **191**:285-291, 2015.

67. Dixit R, Weissfeld JL, Wilson DO, Balogh P, Sufka P, Siegfried JM, Grandis JR, Diergaarde B. Incidence of head and neck squamous cell carcinoma among subjects at high risk of lung cancer: results from the Pittsburgh Lung Screening Study. *Cancer* **121**:1431-1435, 2015.

68. Sanchez-Salcedo P, Wilson DO, de-Torres JP, Weissfeld JL, Berto J, Campo A, Alcaide AB, Pueyo J, Bastarrika G, Seijo LM, Pajares MJ, Pio R, Montuenga LM, Zulueta JJ. Improving selection criteria for lung cancer screening. The potential role of emphysema. *Am J Respir Crit Care Med* **191**:924-931, 2015.

69. Thalanayar PM, Altintas N, Weissfeld JL, Fuhrman CR, Wilson DO. Indolent, Potentially Inconsequential Lung Cancers in the Pittsburgh Lung Screening Study. *Ann Am Thorac Soc* **12**:1193-1196, 2015.

70. Weissfeld JL, Lin Y, Lin HM, Kurland BF, Wilson DO, Fuhrman CR, Pennathur A, Romkes M, Nukui T, Yuan J-M, Siegfried JM, Diergaarde B. Lung Cancer Risk Prediction Using Common SNPs Located in GWAS-Identified Susceptibility Regions. *J Thorac Oncol* **10**:1538-1545, 2015.

71. Wilson DO, Weissfeld J. A simple model for predicting lung cancer occurrence in a lung cancer screening program: The Pittsburgh Predictor. *Lung Cancer* **89**:31-37, 2015.

72. Balkan A, Bulut Y, Fuhrman CR, Fisher SN, Wilson DO, Weissfeld JL, Sciurba FC. COPD phenotypes in a lung cancer screening population. *Clin Respir J* **10**:48-53, 2016.

73. Zhang X, Sebastiani P, Liu G, Schembri F, Dumas YM, Langer EM, Alekseyev Y, O'Connor GT, Brooks DR, Lenburg ME, Spira A. Similarities and differences between smoking-related gene expression in nasal and bronchial epithelium. *Physiol Genomics* **41**:1-8, 2010.

74. Travis W, Brambilla E, Burke AP, Marx A, Nicholson AG. The World Health Organization Classification of Tumours of the Lung, Pleura, Thymus and Heart. Lyon, France: IARC; 2015.

75. Cattoretti G, Becker MH, Key G, Duchrow M, Schluter C, Galle J, Gerdes J. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* **168**:357-363, 1992.

76. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* **133**:1710-1715, 1984.

77. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer* **31**:13-20, 1983.

78. Ooi AT, Gower AC, Zhang KX, Vick JL, Hong L, Nagao B, Wallace WD, Elashoff DA, Walser TC, Dubinett SM, Pellegrini M, Lenburg ME, Spira A, Gomperts BN. Molecular profiling of premalignant lesions in lung squamous cell carcinomas identifies mechanisms involved in stepwise carcinogenesis. *Cancer Prev Res (Phila)* **7**:487-495, 2014.

79. Sridhar S, Schembri F, Zeskind J, Shah V, Gustafson AM, Steiling K, Liu G, Dumas YM, Zhang X, Brody JS, Lenburg ME, Spira A. Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. *BMC Genomics* **9**:259, 2008.

80. Frison L, Pocock SJ. Repeated measures in clinical trials: analysis using mean summary statistics and its implications for design. *Stat Med* **11**:1685-1704, 1992.

81. Quan H, Zhang J. Estimate of standard deviation for a log-transformed variable using arithmetic means and standard deviations. *Stat Med* **22**:2723-2736, 2003.

82. Whitney DH, Elashoff MR, Porta-Smith K, Gower AC, Vachani A, Ferguson JS, Silvestri GA, Brody JS, Lenburg ME, Spira A. Derivation of a bronchial genomic classifier for lung cancer in a prospective study of patients undergoing diagnostic bronchoscopy. *BMC Med Genomics* **8**:18, 2015.

83. Liu P, Hwang JT. Quick calculation for sample size while controlling false discovery rate with application to microarray analysis. *Bioinformatics* **23**:739-746, 2007.