Protocol Title	Clinical Trial of Watercress in Detoxification of Environmental Toxicants and Carcinogens
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PROTOCOL COVER PAGE

REVISION HISTORY

Revision #	Version Date	Summary of Changes	Consent Change?
1	12/3/19	Made changes to study product formulation; Revised the protocol to allow for guidelines set forth by the Center for Disease Control and the University of Minnesota to move any study procedures that can be to a virtual status.	Y
2	7/23/20	Changed day 2 to day 4 in schedule of events	Y

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ABBREVIATIONS/DEFINITIONS

AE: Adverse Event
BP: Blood Pressure
BPM: Beats per Minute
CVD: Cardiovascular Disease
DAST: Drug Abuse Screening Test
DSMB: Data Safety Monitoring Board
DVT: Deep Vein Thrombosis
GSTM1: Glutathione-S-transferase M1
GSTT1: Gluthathione-S-transferase T1
HMPMA: Abbreviation of mercapturic acid metabolite
HPMA: Abbreviation of mercapturic acid metabolite
HR: Heart Rate
IRB: Institutional Review Board
IUD: Intrauterine Device
MAR: Missingness at Random
MI: Myocardial Infarction (heart attack)
NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-buatonone
O2: Oxygen
PE: Pulmonary Embolism
PEITC: 2 PHENENTHYL ISOTHIOCYANATE
PTCA: Angioplasty/stenting
SAE: Serious Adverse Event
SMAST: Michigan Alcohol Screening Test Short
SPMA: Abbreviation of mercapturic acid metabolite

1.0 Objectives

The goal of this proposal is to test the hypothesis that consumption of watercress, a rich source of the cancer chemopreventive agent 2-phenethyl isothiocyanate (PEITC), will enhance the detoxification of multiple environmental toxicants and carcinogens, particularly in subjects who are null for *glutathione-S-transferase M1* (*GSTM1*), *glutathione-S-transferase T1* (*GSTT1*), or both. If our hypothesis is supported by the results of the trial, watercress could emerge as an inexpensive and plentiful dietary constituent which could promote good health by decreasing the effects of environmental stressors.

We and others have carried out extensive studies on the efficacy and mechanisms of PEITC in the context of cancer chemoprevention [1-21] Our studies focused on the potential use of PEITC in prevention of lung cancer in smokers, as we have demonstrated that PEITC is a powerful inhibitor of lung carcinogenesis in rats and mice treated with the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [2,20]. These studies led to a clinical trial of PEITC with the goal of determining whether it could inhibit the metabolic activation of NNK, as observed in rats and mice [11]. The results of that trial demonstrated a modest but significant effect of PEITC in decreasing the metabolic activation of NNK in cigarette smokers [11].

Unexpectedly however, PEITC showed far greater effects on the detoxification of benzene, acrolein, and crotonaldehyde in subjects who were null for *GSTM1*, *GSTT1*, or both [21] (see Preliminary Results). We now wish to extend these exciting results and assess the effects of watercress, as an abundant source of PEITC as well as other isothiocyanates, on the detoxification of environmental toxicants and carcinogens in a trial that will recruit *both* non-smokers and smokers in proportion to their prevalence in the Minnesota population. All humans are exposed to benzene, acrolein, crotonaldehyde and other related toxicants and carcinogens through their diet, polluted air, and/or endogenous metabolic sources.

2.0 Background

Volatile organic carcinogens and toxicants such as those summarized in **Table 1** are ubiquitous environmental and endogenous compounds to which virtually all humans are exposed. All of these compounds are detoxified by metabolic processes that ultimately result in conjugation with glutathione and excretion of mercapturic acids in urine [22]. Glutathione conjugation can be upregulated by isothiocyanates through the Nrf2 pathway and related routes of metabolism [23]. The hypothesis underlying this proposal is that watercress consumption, resulting in exposure to milligram amounts of PEITC per day, will enhance the detoxification of these and related compounds. As summarized in **Table 1**, three of these compounds – benzene, ethylene oxide, and 1,3-butadiene – are classified as "carcinogenic to humans" by the International Agency for Research on Cancer.

Benzene causes acute myeloid leukemia/acute non-lymphocytic leukemia in humans, and a positive association has been observed between benzene exposure and acute lymphocytic leukemia, chronic lymphocytic leukemia, multiple myeloma, and non-

Hodgkin lymphoma. Ethylene oxide is believed to be a cause of lymphatic and hematopoietic cancers as well as breast cancer, and there is strong evidence that it operates by a genotoxic mechanism in humans. 1,3-Butadiene causes cancer of the hematolymphatic organs, and there is also strong evidence that it operates by a genotoxic mechanism in humans, via formation of epoxides. Glutathione conjugation interferes with these genotoxicity pathways. Acrolein and crotonaldehyde are intense eye and respiratory tract irritants which cause a variety of toxic effects including irritation, inflammation, and cell proliferation [24]. Both are DNA damaging compounds and, although lacking strong carcinogenic activity, acrolein-DNA binding has been implicated in lung cancer etiology because its pattern of DNA damage in the p53 gene is similar to that observed in lung cancer [25]. Propylene oxide has been classified as "possibly carcinogenic to humans" [26]. Acrylonitrile causes tumors of the central nervous system in rats and has also been evaluated as 'possibly carcinogenic to humans" [27]. Acrylamide has attracted considerable attention because of its presence in certain cooked foods. Although it is genotoxic, there is currently uncertainty concerning its contribution to the occurrence of human cancers [28]. The structures of the mercapturic acids which are formed from these compounds and which will be analyzed in this project are shown in **Table 1**. We will also perform a more extensive analysis of urinary mercapturic acids in our study subjects. Significant increases in the detoxification of these ubiquitous volatile environmental toxicants and carcinogens, as indicated by urinary mercapturic acid levels, would indicate protection against their toxic and carcinogenic effects, which could be important in prevention of cancer and other diseases.

Table 1. Specific carcinogens and toxicants to be considered in this proposal

Compound	IARC evaluation class[a]	Structure of mercapturic acid metabolite R-S-$CH_2CH(NHAc)COOH$ R =	Abbreviation of mercapturic acid metabolite	Ref.
Benzene	1	C_6H_5	SPMA	[29]
Acrolein[b]	3	$(CH_2)_3OH$	3-HPMA	[30]
Crotonaldehyde	3	$CH_3CH(CH_2)_2OH$	HMPMA	[31]
Propylene oxide	2B	$CH_2CHOHCH_3$	2-HPMA	[26]
Acrylonitrile	2B	CH_2CH_2CN	CEMA	[27]
Ethylene oxide	1	CH_2CH_2OH	HEMA	[29]
1,3-Butadiene	1	$CH_2CHOHCH=CH_2$	MHBMA	[29]
Acrylamide[c]	2A	$CH_2CH_2CONH_2$	AAMA	[32]

[a] 1, Carcinogenic to humans; 2A, probably carcinogenic to humans; 2B, possibly carcinogenic to humans; 3, not classifiable as to its carcinogenicity to humans
[b] Evaluated before evidence of its selective binding at *p53* hotspots
[c] Evaluated prior to evidence of its occurrence in certain foods

As described under "Preliminary Studies", we have observed a significant enhancing effect of PEITC consumption on the detoxification of benzene and acrolein, as determined by analysis of their urinary mercapturic acids SPMA and 3-HPMA [21]. The observed effects were particularly strong in subjects lacking the *GSTM1* or *GSTT1* genes, or both, and were also observed for detoxification of crotonaldehyde

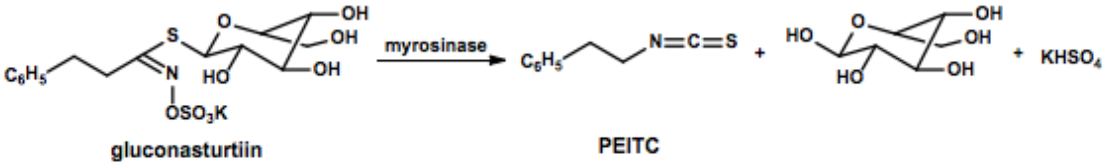
(as determined by HMPMA in urine) in these individuals. Similar results were obtained in a clinical trial of a "broccoli sprouts tea" beverage, rich in the isothiocyanate sulforaphane, carried out in China by the Kensler group [33]. In that study, in which the urinary mercapturic acid analyses were performed by our laboratory, rapid and sustained statistically significant increases in the levels of excretion of SPMA from benzene (61% increase) and 3-HPMA from acrolein (23% increase) were observed, although there was no statistical effect of *GSTM1* and *GSTT1* status on the results. Collectively, these results clearly demonstrate that isothiocyanates, whether administered in pure form, as in the PEITC clinical trial, or in the form of a vegetable beverage, as in the broccoli sprouts clinical trial, can enhance detoxification of environmental toxicants and carcinogens. We also note that in the latter trial, exposure to benzene and acrolein was remarkably higher than in the U.S. because of significant levels of air pollution in the Shanghai area where the trial was performed. Collectively, these results strongly support our hypothesis that watercress consumption can enhance the detoxification of volatile organic carcinogens and toxicants, thus enhancing health by providing protection against the effects of environmental toxicants and carcinogens. Our results could significantly expand the armamentarium of cruciferous vegetables with demonstrated protective effects against carcinogens and toxicants.

Epidemiologic studies provide some evidence for the role of cruciferous vegetables and isothiocyanates in protection against several cancers [34]. A meta-analysis of greater than 8000 lung cancer cases and 684,000 non-cancer subjects showed an overall 20% lower risk of lung cancer associated with cruciferous vegetable intake, with the strongest risk reduction in subjects who were *GSTM1* or *GSTT1* null, and particularly in the double nulls (odds ratio = 0.4, 95% CI, 0.20-0.68) [35]. The protective effect of cruciferous vegetable consumption against lung cancer was observed in both smokers and non-smokers. The greater risk reduction in the *GSTM1* and *GSTT1* nulls has been attributed to inhibition of the metabolism and excretion of isothiocyanates, but our clinical trial data as well as other published data do not support this hypothesis as *GSTM1* and *GSTT1* genotypes had no significant effect on urinary levels of the mercapturic acid PEITC-NAC, the principle metabolite of PEITC [21,36]. Thus, the mechanistic basis for the greater protective effect of isothiocyanates in *GSTM1* and *GSTT1* null individuals remains unclear, although we hypothesize that it could be due to induction of *GSTP1*, which we propose to test in later years of this program.

In this proposal we focus on watercress as the source of PEITC rather than PEITC itself, which was used in our completed trial. The study of pure PEITC as a chemopreventive agent against lung cancer has been an instructive experience for our research team. Our initial studies showing that PEITC inhibited the metabolic activation of certain carcinogens were published over 30 years ago [37]. There are many challenging steps required to bridge the gap between efficacy studies in laboratory animals and human clinical trials. We have overcome numerous difficulties, both technical and financial, in bringing PEITC to a clinical trial and completing that trial [11]. One of the most vexing issues was formulation of PEITC, an oil, into softgels. The expense of doing this under the required GMP conditions

and on the scale needed for the clinical trial was prohibitive for an academic laboratory. The overall experience with PEITC has convinced our team that using a common vegetable as a source of the same agent is more economical and practical. Furthermore, we strongly support the thesis put forward by Fahey et al concerning "green chemoprevention as frugal medicine [38]." The use of specific foods rather than pure compounds derived from these foods for health maintenance and cancer prevention is not only credible scientifically and based on substantial evidence from published studies, but also more likely to have wide applicability in a global setting [38].

Watercress is a uniquely abundant source of gluconasturtiin, the glucosinolate precursor to PEITC [39]. The structure of gluconasturtiin (β-phenethyl glucosinolate) is shown in **Figure 1**. Glucosinolates are characteristic components of cruciferous vegetables. They are plant defense compounds. When cells of the plant are broken, as in chewing of the vegetable, the enzyme myrosinase which is normally cellularly separated from the glucosinolates, is released and mixes with the glucosinolate resulting in conversion of the glucosinolate to an isothiocyanate, as shown for PEITC in **Figure 1**. Multiple studies including our own have shown that gluconasturtiin is a major glucosinolate in watercress, generally exceeding all other glucosinolates and that watercress is the main natural source of this compound [34,39-41]. Thus, consumption of about 10g of watercress (wet weight) will result in uptake of about 2 mg of PEITC, according to our current preliminary data. Watercress also contains other bioactive glucosinolates such as 7-methylsulfinylheptyl-, 8-methylsufinyloctyl-, and glucobrassican (the precursor to indole-3-carbinol), although these are generally found in considerably lower quantities than gluconasturtiin [34,39-41].



gluconasturtiin PEITC

Figure 1. Formation of PEITC from gluconasturtiin

Several small trials have examined the effects of watercress consumption on endpoints associated with cancer prevention. We showed that consumption of watercress enhanced the excretion of the NNK metabolite NNAL in smokers, suggesting inhibition of its metabolic activation, as observed in studies of PEITC and NNK in laboratory animals [42]. Gill et al demonstrated that consumption of watercress (85 g daily for 8 weeks) was associated with reductions in DNA damage, with a greater affect in smokers than non-smokers [43]. Syed Alwi et al found that watercress consumption reduced 4E-BP1 phosphorylation leading potentially to inhibition of hypoxia-inducible factor, a key transcription factor involved in cellular responses to O_2 [44]. Other studies have obtained various results in the examination of watercress extracts in cell culture systems [41,44-46].

Since the initial studies by Chung et al showing that PEITC inhibited the metabolic activation of NNK [37], multiple investigations have confirmed and extended these results to demonstrate favorable properties of non- toxic doses of PEITC in a variety

of cancer prevention settings, initially focused on mechanisms associated with inhibition of carcinogen metabolic activation and tumor induction, but then extended by numerous investigators to include multiple effects on important components of the tumor induction process [6,9,12,16,19,34,47-54]. These studies leave little doubt about the cancer chemoprevention properties of PEITC. Thus, using watercress as a source of PEITC and other bioactive glucosinolates, as proposed here, is a practical, promising, and potentially significant approach to enhancing detoxification of environmental carcinogens and toxicants.

Relatively few clinical studies have examined the efficacy of food-based preparations in cancer prevention. Some notable examples have been summarized recently [38] and include studies on powdered freeze-dried berries [55], pomegranate [56], tomato [57], broccoli sprouts [33], tea [58], garlic [59], and ginger root [60]. There is one clinical trial of watercress supplementation for modulation of cancer progression and disease recurrence listed on clinical trials.gov. This is quite different from our proposed trial which focuses on prevention. Thus, our proposed clinical trial of watercress is innovative, especially when combined with our analytical chemistry and mass spectrometric expertise which will allow the quantitation of effects on a wide range of potentially carcinogenic and toxic environmental chemicals. The great potential of watercress and its constituent isothiocyanates to enhance the detoxification of environmental toxicants and carcinogens has not been sufficiently investigated in previous studies.

One group of people routinely exposed to high levels of volatile toxic combustion products is firefighters. We will work with firefighters to increase awareness of our approach and have contacted Mr. Ken Bence in this regard. Watercress consumption has the potential to ameliorate the toxic effects of volatile combustion products such as acrolein and benzene to which firefighters are exposed.

Preliminary Studies

Effects of PEITC on detoxification of benzene, acrolein, and crotonaldehyde

The results of the clinical trial of PEITC that form the basis of this proposal have been described [21]. The study was a randomized, placebo-controlled, double-blind phase II clinical trial with a crossover design. Current smokers of 10-45 cigarettes per day who were 21 years or older and in good health were enrolled. For reasons unrelated to this proposal, the subjects smoked commercial cigarettes with added [pyridine-D4]NNK during the study. After 2 weeks of adaptation to smoking these cigarettes, the smokers were randomly assigned to either the PEITC then placebo arm or the placebo then PEITC arm of the trial. During the treatment period, each subject was asked to take PEITC (10 mg in 1 ml olive oil) 4 times/day, once every 4 h, for five days (week 3 or 5), or the placebo agent (olive oil) on the same schedule (week 3 or 5). There was a one week washout period between the PEITC and placebo treatments (week 4). Twenty-four h urine samples were collected at the end of the smoking adaptation period (week 2), on 3 days (3rd, 4th, and 5th day) of each of the two treatment periods (weeks 3 and 5), and at the end of the washout period (week 4). Blood and buccal cell samples were collected collected at baseline, and at the end

of the smoking adaptation period, each of the two treatment periods, and the washout period. Forty-one subjects in the PEITC-placebo group and 41 subjects in the placebo-PEITC group completed the study. Their urine samples were analyzed for SPMA, 3-HPMA, and HMPMA, mercapturic acids derived from benzene, acrolein, and crotonaldehyde, respectively, using validated methods [61,62].

Table 2. Geometric means (95% confidence intervals) of baseline urinary mercapturic acid metabolites of benzene, acrolein and crotonaldehyde, in smokers separated by *glutathione S-transferase* (*GST*) *M1* and *T1* genotypes.

GST genotype	N[a]	Benzene SPMA (pmol/mg Cr)[b]	Acrolein 3-HPMA (nmol/mg Cr)[b]	Crotonaldehye HMPMA (nmol/mg Cr)[b]
GSTM1				
Null	36	1.72 (1.22-2.40)	7.12 (5.98-8.48)	5.16 (4.22-6.28)
Present	45	3.10 (2.28-4.20)	6.28 (5.36-7.36)	4.28 (3.58-5.12)
P		0.014	0.309	0.186
GSTT1				
Null	19	0.80 (0.52-1.18)	7.22 (5.64-9.22)	5.26 (3.98-6.94)
Present	62	3.34 (2.68-4.18)	6.48 (5.66-7.40)	4.48 (3.86-5.22)
P		<0.001	0.459	0.338
GSTM1 & GSTT1				
Both null	12	0.44 (0.28-0.74)	8.58 (6.26-11.74)	5.76 (4.02-8.26)
One present	31	2.84 (2.12-3.82)	6.28 (5.22-7.58)	4.82 (3.88-5.96)
Both present	38	3.56 (2.74-4.66)	6.40 (5.40-7.58)	4.22 (3.48-5.12)
P for trend		<0.001	0.244	0.130

[a] One subject who did not provide a urine sample at baseline was excluded from all analyses.
[b] All geometric means were adjusted for age and gender.

As shown in **Table 2**, *GSTT1* and *GSTM1* status had a strong effect on levels of SPMA from benzene, but not on levels of 3-HPMA or HMPMA from acrolein and crotonaldehyde, respectively. This is consistent with previous results [62-64].

Among all subjects, intake of PEITC significantly increased the urinary excretion of SPMA by 24.6% (P=0.002) and 3-HPMA by 15.1% (P = 0.005), but did not have a significant effect on levels of HMPMA. But as shown in **Table 3**, larger effects were observed when the subjects were classified by *GST* genotype.

Table 3. The effect of PEITC on the changes of urinary concentrations of merpcaturic acid metabolites of benzene, acrolein and crotonaldehyde in smokers stratified by the *glutathione S-transferase (GST) M1* and *T1* genotype, The PEITC Intervention Study 2008-2013.

GST genotype	*N*	Geometric mean		% difference (95% CI)	*P*[a]	*P* for interaction[b]
		Placebo	PEITC			
GSTM1		Benzene SPMA (pmol/mg creatinine)				
Null	37	2.09	2.99	43.1 (18.1, 73.4)	<0.001	0.060
Present	45	3.12	3.47	11.2 (-6.5, 32.4)	0.234	
GSTT1						
Null	19	0.83	1.45	74.2 (33.9, 126.6)	<0.001	0.006
Present	63	3.67	4.14	12.7 (-2.4, 30.4)	0.108	
GSTM1 & GSTT1						
Both null	12	0.74	1.45	95.4 (40.7, 171.5)	<0.001	0.009
One present	32	2.64	3.36	27.4 (4.3, 55.6)	0.020	
Both present	38	3.84	4.07	6.1 (-11.6, 27.5)	0.526	
GSTM1		Acrolein 3-HPMA (nmol/mg creatinine)				
Null	37	8.07	10.05	24.6 (7.9, 43.8)	0.005	0.179
Present	45	6.79	7.37	8.6 (-4.3, 23.2)	0.210	
GSTT1						
Null	19	8.00	10.12	26.6 (5.4, 52.0)	0.022	0.320
Present	63	7.15	8.00	12.0 (-0.1, 25.6)	0.057	
GSTM1 & GSTT1						
Both Null	12	9.84	13.06	32.7 (5.9, 66.2)	0.034	0.315
One present	32	6.90	8.28	20.0 (1.8, 41.3)	0.038	
Both present	38	7.04	7.53	7.0 (-6.9, 22.9)	0.347	
GSTM1		Crotonaldehyde HMPMA (nmol/mg creatinine)				
Null	37	5.89	6.80	15.4 (3.9, 28.1)	0.009	0.027
Present	45	4.62	4.53	-1.9 (-10.8, 7.8)	0.686	
GSTT1						
Null	19	5.34	6.52	22.0 (5.3, 41.4)	0.010	0.031
Present	63	5.10	5.15	1.0 (-6.8, 9.5)	0.808	
GSTM1 & GSTT1						
Both Null	12	6.19	8.04	29.8 (8.2, 55.8)	0.006	0.017
One present	32	5.36	5.85	9.2 (-2.3, 22.0)	0.124	
Both present	38	4.71	4.53	-4.0 (-13.3, 6.3)	0.438	

[a] 2-sided *P* values were derived from the mixed models that test PEITC treatment effect on the change of urinary levels of biomarkers within each specific *GST* genotypes before and after PEITC intake.

[b] 2-sided *P* values were derived from the mixed models that test the interaction term between PEITC intake and *GST* genotype on the levels of urinary biomarkers.

Thus, for SPMA, PEITC caused significant 43.1%, 74.2%, and 95.4% increases in SPMA in the subjects who were *GSTM1* null, *GSTT1* null, and *GSTM1* plus *GSTT1* null, respectively. For the acrolein metabolite 3-HPMA, the corresponding figures were 24.6%, 26.6%, and 32.7%, all statistically significant, while for the crotonaldehyde metabolite HMPMA statistically significant increases of 15.4%, 22.0%, and 29.8% were seen in the *GSTM1* null, GSTT1 null, and GSTM1 plus GSTT1 nulls, respectively. Thus, GST status had a remarkable effect on the ability of PEITC to enhance detoxification of benzene, acrolein, and crotonaldehyde. This was particularly evident for SPMA in the nulls, because these individuals have relatively low basal levels of SPMA, although the increases still did not raise their levels above those of non-nulls. We hypothesize that the significant increases in mercapturic acids observed upon treatment with PEITC is due to induction by PEITC of GSTP1, which is an established catalyst of these reactions, but further studies are required, as induction of GSTP1 by isothiocyanates appears to be complex [65,66]. These studies are beyond the scope of the present proposal, but we will examine this hypothesis in the future, pending the results of the clinical trial proposed here.

Analysis of Watercress for Gluconasturtiin and PEITC

We purchased watercress from a local market, divided it into portions, and freeze dried it. We analyzed each for gluconasturtiin using a published HPLC method which we described previously [40]. The average level of gluconasturtiin was 2 μmol per gram wet weight watercress. In an important new experiment, we determined that when this freeze-dried watercress was placed in H2O for 10 min, PEITC (2 μmol/g wet weight watercress) was released. The gluconasturtiin in this freeze-dried watercress was stable in a -20 °C freezer for at least 9 months. This experiment provides a clear pathway for dose formulation in the clinical trial. Watercress can be purchased and freeze-dried, conditions under which there is no loss of gluconasturtiin because of its relatively high molecular weight (423 g/mol). This freeze-dried watercress is stable at -20 °C. When this freeze-dried watercress is added to H2O, an equivalent amount of PEITC is released within 10 min because the cells in the watercress have been broken during freeze-drying which allows myrosinase to mix with gluconasturtiin when the freeze-dried powder is placed in H2O, thus releasing an equivalent amount of PEITC.

3.0 Study Endpoints/Events/Outcomes

The primary endpoint is to conduct a clinical trial with 350 subjects (both non-smokers and smokers) to determine the effects of the watercress preparation on detoxification of environmental toxicants and carcinogens.

4.0 Study Intervention(s)/Investigational Agent(s)

We will freeze-dry approximately 2000 lbs of watercress to produce a powder (about 100 lbs, or 45.4 kg). Freeze-drying on the large scale required for this study will be performed by Van Drunen Farms. The resulting powder will contain the gluconasturtiin precursor to about 3 mg PEITC per gram (which will be released upon addition to H_2O.) Subjects will come to the clinic and receive a pre-filled sealed jar of

freeze-dried watercress powder and a pre-packaged flavoring product, which they will add to water and stir. Each jar of freeze-dried watercress (FDWC) will contain 125 g, two jars will be given to each participant for a total of 250 g. Dosing calculations will vary depending on the potency of PEITC for each batch of FDWC (a small sample will be taken from each batch and potency will be measured). An example calculation is as follows: a total of forty-two 4.4-gram portions will be taken over the 2-week period; each 4.4 gram portion will release about 13.2 mg of PEITC. The 2-week supply will provide for an extra 14 doses for a total of 56 doses (4.4 x 56 = 246). For the placebo, we plan to use maltodextrin, which is a polysaccharide and does not contain glucosinolates, and the same flavoring product used to prepare the watercress drink. Subjects will prepare the placebo drink in the same way as the watercress drink. Each jar of maltodextrin will contain 250 g, one jar will be given to each participant. A total of forty-two 4.4-gram portions will be taken over the 2-week period and each container will hold an extra 14 doses for a total of 56 doses (4.4 x 56 = 246).

Flavoring Product

We will use a commercially available flavoring product such as Crystal Light or SweetLeaf Water Drops™. Participants will mix a pre-determined amount of flavoring product with the FDWC or placebo.

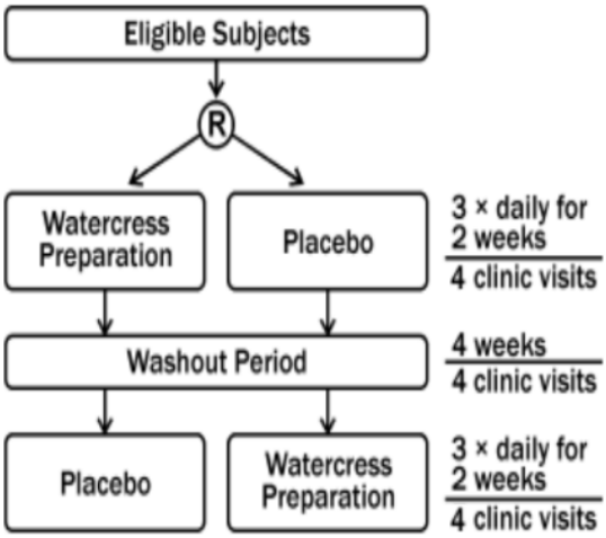
Drink Preparation

Freeze-dried Watercress. Participants will be instructed to mix one scoop (73 mL) of FDWC with 16 oz of water and stir for 30 seconds, then let the mixture rest for 2 minutes. Next, they will add in the flavoring product and stir for 10 seconds. Participants will be asked to finish the drink within 10 minutes of mixing .

Maltodextrin. Participants will be instructed to mix one scoop (11 mL) of maltodextrin with 16 oz of water and stir for 30 seconds, then let the mixture rest for 2 minutes. Next, they will add in the flavoring product and stir for 10 seconds. Participants will be asked to finish the drink within 10 minutes of mixing.

5.0 Procedures Involved

The study will be a randomized, placebo-controlled, single-blind, phase II clinical trial with a crossover study design. Participants will be assigned to active or placebo study product for 14 days, then undergo a 4 week wash-out period, and will then be crossed over to the other product for another 14 days (see **Figure 2**). During the treatment phase, subjects will consume the watercress beverage or placebo, 3 times per day. The target dose will be 40 mg/day of PEITC, as in our previous study. Urine, oral swabs, saliva, and blood will be collected.



Screening Visit

This study will be conducted at two Minnesota sites: University of Minnesota Twin Cities and Hormel Institute, which is a part of the University of Minnesota. Subjects will first undergo a prescreening visit, which will be conducted via phone or online REDCap survey. Those who meet the eligibility criteria will be invited to complete a virtual screening visit (completed via secured video-conferencing link). In order to follow guidelines set forth by the Centers for Disease Control and the University of Minnesota, a significant portion of the study procedures will be completed remotely.

During the virtual screening visit, we will explain, that in the trial, subjects will be asked to use a drink prepared from watercress or an inert substance (placebo) that will be provided by us. The order in which they receive the watercress or placebo product will be randomized. Each product will be used for a period of 14 consecutive days with a 4-week interval separating the two periods. An electronic informed consent (e-consent) will then be obtained. Following consent, study participants will be asked to complete questionnaires to assess demographics, medical and medication history, psychiatric status and substance abuse history (see **Table 4**). Our Licensed Medical Professional, will review these health questionnaires for consistency and eligibility for the study. Tobacco users will be asked to complete a tobacco use history form that assesses amount and frequency of tobacco use. Eligible participants will then be scheduled for a clinic visit and asked to refrain from eating any cruciferous vegetables during the course of the study. (Cruciferous vegetables are the only other major source of isothiocyanates that could affect the study results.) A list and pictures of cruciferous vegetables will be provided to each participant. Participants will be provided a urine cup (via mail) and asked to collect their first morning urine on the day of their next clinic visit. A reminder call or text will be sent to the participant prior to this visit.

Experimental Period

Study design. Participants will be asked to attend clinic visits on days 0 (the day of product distribution), 4 (±1 day), 7 (±1 day) and 14 (±1 day) for each of the periods they are receiving product and weekly during the 4 week washout between periods (see **Figure 2**). We use a 4-week washout period because the 1-week washout in our preliminary studies may not have been sufficient [11]. Each visit is composed of three parts and will be completed in a single day. Part 1: Prior to the clinic visit the participant will collect their first morning void urine. Part 2: In a virtual visit, participants will be asked to complete multiple online surveys (see **Table 4**). Part 3: Participants will drop off their first morning void urine (also used to test for pregnancy) at the research clinic (University of Minnesota – Tobacco Research Programs, 717 Delaware St. SE). Blood (once allowed), blood pressure, saliva, oral rinse, and buccal cells will be collected. After this collection, participants will be provided with either the watercress powder or the placebo and the flavoring product. Subjects will be given written instructions on how to add the watercress/placebo powder and flavor product to water and will also be provided a link to a video with instructions. They will be provided three different flavors from which they can sample. At the Day 4 visit, they will be asked to choose one of the flavors to be used during the rest of this phase. They will be asked to drink the formulation at breakfast, lunch and dinner. Participants will receive one jar of the FDWC on day 0 and the second jar on day 7. Participants will receive one jar of maltodextrin on day 0, which will last until the end of the study. A log will be kept of the products that are dispensed and any amounts unused. At the end of each two-week period (or end of study participation, whichever comes first), we will ask all participants to return any unused product. Compliance of use of the study products will be determined by the product accountability log and biochemical verification (e.g., urinary PEITC-NAC and total isothiocyanates). Participants will be provided standardized counseling about the importance of using the assigned products as directed and if procedural compliance is an issue, the research assistant will discuss the obstacles and develop a plan to overcome these obstacles. Participants will also be provided a urine collection cup, dietary intake log and dosing log at each visit.

Biospecimen collection. All collection containers will be labeled with study number, subject number, collection date and type of biological sample (urine, blood, saliva, buccal cells). At each clinic visit, first morning void urine samples will be collected and participants will be asked to submit saliva and oral cells. Participants will be asked to brush their teeth in the bathroom at the beginning of the visit by using a provided commercial individually packed pre-pasted toothbrush (ReadyBrushTM) and asked to not eat, drink, smoke or chew gum for 5 min prior to obtaining oral cells. Saliva samples will be collected by asking the participant to spit in a sterilized 10 ml tube. These samples will be frozen at -20 °C for possible future oral microbiome studies. Oral cells will be collected by first, doing an oral rinse. Participants will squirt 15 mL of saline solution into their mouths and swish for 45 seconds. The oral rinse sample will be expectorated back into a 50 mL tube. After completing the rinse, participants will scrape the oral mucosa inside the mouth with a cytobrush. Two cytobrushes will be used on each subject to collect two separate samples from the left and the right inner cheek. After scraping the mucosal surface, each cytobrush will be immediately placed into a separate pre-labeled sterile polypropylene tube containing 5 mL of saline, swirled for a

few seconds, and tapped against the walls of the tube to ensure the cells are transferred from the brush to the saline. Oral cells will be stored at -20°C for DNA isolation and research in future years of this study, which may include analysis for protein expression, including GSTP1. The use of oral cells for protein expression studies has been described [70].

Blood collection. Blood will not be collected until allowed per University policy. Once allowed by the University, blood collection will occur in two phases. Phase 1: One tube of blood will be collected (8 mL purple top). Depending on when blood collection is allowable, it will be collected on day 0 or if the participant has already completed the study, we will contact them again to have them come to the clinic for a one time blood draw. Phase 2: Blood will be collected on days 0, 7 and 14. Two tubes of blood (8 mL each, purple top) will be drawn by a trained technician. Blood samples will be used for DNA and protein isolation. The DNA will be used for genotyping (described under Specific Aim 3) and the protein reserved for possible future studies of expression of GSTP1 and other proteins. Protein will be isolated from peripheral blood mononuclear cells as described [69].

Sample preparation Blood collected into purple top tubes will be processed within 12 h of phlebotomy. Samples should remain on ice until processing. The tubes will be centrifuged at 2500 × g for 15 min in a refrigerated centrifuge to separate plasma, buffy coat, and red blood cells. The separated components will be transferred into cryogenic vials, labeled (with information described above) and stored in a -80 °C freezer. For genetic analysis, genomic DNA is purified from buffy coats using a PureGene Blood Kit (Gentra Systems, Minneapolis, MN) according to the instructions provided by the manufacturer. DNA samples are aliquoted into 96-well plates using robotic equipment to minimize human error. The extracted DNA samples will be used for the determination of *GST* genotypes. The urine samples will be aliquoted into cryotubes and frozen at -20 °C. Tubes with buccal cell samples will be centrifuged at 2500 × g for 15 min to pellet the cells and the supernatant will be discarded. The cell pellets will be washed by adding 1 mL of pH 7.4 buffer, swirling the tube gently, repeating the centrifugation, and discarding the buffer. The washed buccal cell pellet will be re-suspended in 100 µL cold buffer, transferred into a pre-labeled cryovial, and stored at -20 °C. Throughout the study, biological samples (blood, buccal cells, urine, saliva) will be collected, labeled and registered on our Biomarker Website. The Hormel Institute will send batches to the Masonic Cancer Center, University of Minnesota, to be stored in the Biorepository with de-identified information. The Hormel Institute and Masonic Cancer Center Biorepositories have established policies and procedures consistent with NCI guidelines. Samples will be stored until fully used, no longer usable, or destroyed upon subject request. A discussion of the storage, future use, and sharing of samples will be presented in the informed consent for this study. A Data and Biospecimen Sharing Plan is also provided below.

Other measures Health changes, medication use, and adverse events since the last visit will be determined, vital signs measured and ingestion of any cruciferous vegetables

assessed. Among tobacco users, specific type of tobacco product use and amounts of use will be determined using the timeline follow-back method.

Table 4. Procedures for each visit

	Pre-Screening	Screening	Clinic visits during the 14 days of study product use (days 0,4,7,14)	Clinic visits (weekly) during one-month wash-out period
Telephone screening (remote)	X			
Consent form (remote)		X		
Screening questionnaires (remote)[a,b]		X		
Body Mass Index (remote)		X		
First urine void			X	X
Urine Pregnancy Test (in-person clinic)		X		
Blood draw (in-person clinic)[c]			X	X
Oral cell collection (in-person clinic)			X	X
Saliva collection (in-person clinic)			X	X
Vital signs (in-person clinic)		X	X	X
Health changes (remote)			X	X
Adverse effects (remote)			X	X
Medications questionnaire (remote)			X	X
Affect liking scale (remote)[d]			X	
Dietary intake Log (remote)			X	X
Study product dose log (remote)			X	
Dispense study products (in-person clinic)			X	

a Demographics, Medical History; Medications Questionnaire, PRIME MD(b), Michigan Alcohol Screening Test(b), Drug Abuse Screening Test(b), and Tobacco Use History for those who are tobacco users.

b Questionnaires will only be administered if the participant responds 'yes' to related items in the Medical History Questionnaire

c Blood will be only be drawn once allowable by the University.

d Affect liking scale will be done on Day 14 for each study product use period

Description of Measurements at the Research Clinic

Screening measures. 1) D*emographics* such as age and gender, race, ethnicity, income, current occupation and usual occupation; 2) *Medical history and current health status* for current diagnoses, symptoms and past health problems; 3) *Medication*s *Questionnaire* to rule out medications that affect the metabolism of targeted constituents; 4) *Prime MD* for depression and anxiety symptoms [71]; 5) *Michigan Alcohol Screening Test Short form (SMAST) [73]*; and 6) *Drug Abuse Screening Test (DAST) [74]*. For those who are tobacco users, we will administer a Tobacco Use History that assesses amount and frequency as well as duration of tobacco use [11]. The following measures comprise the *Biomarker modifier questionnaires* (measures of factors that may moderate biomarkers at each clinic visit): 1) *Dietary Intake* assesses consumption of cruciferous vegetables since the prior visit; 2) *Medications Questionnaire* to assess any changes in medications taken since last visit and to determine use of any drugs that may affect the Nrf2 pathway; 3); *Safety measures*: *Health Changes and Adverse Events Scale* to assess the nature, severity, duration, action taken, and outcome of any adverse events. *Vitals:* Blood pressure and heart rate will be measured.

Information about each subject will be entered into a database by the Study Coordinator. Each subject will be coded with a unique number, and only these coded ID's will be entered into the database. All raw data will be kept in locked file cabinets. Only the Project Manager, Study Coordinator and Principal Investigators will have access to individually identifiable private information about participants. Coded ID's will be used throughout the study by all the researchers involved. While all the samples and information will be collected specifically to achieve the goals of this proposal, de-identified individual subject data and back-up samples may be available to other researchers for research purposes after our study is complete. Permission will be obtained from participants to allow de-identified biosamples to be stored in a biorepository for future analyses of biomarkers or genotyping. Standard operating procedures will be developed, as in our previous studies. A tracking database will be developed to follow each participant from enrollment to study completion. A detailed study manual will be developed with step-by-step instructions for interviewers and phlebotomists. All staff will be trained in the procedures and visits will be made to the Hormel Institute site to monitor the case report books and ensure that the protocol is strictly followed.

6.0 Data and Specimen Banking

Biomarker specimens will be collected and stored at the study sites (University of Minnesota - Tobacco Research Programs, 717 Delaware St. SE and Hormel Institute – 801 16th Ave NE, Austin, MN) until delivery to the Masonic Cancer Center's Dr. Stephen Hecht's laboratory for storage and analysis. Samples that are not used for the primary analysis of study biomarkers will be banked for future use. The banked samples will be stored until analyses and destroyed if it is determined they are no longer needed. The samples, which may also include DNA or RNA, may be stored up to a maximum of 10 years from the study's end. A subject has the right to withdraw consent at any time by informing the Principal Investigator by following the instructions provided in the consent

and HIPAA documents. If this occurs, any remaining identifiable research sample(s) will be destroyed.

Data will be stored in a secure database (RedCap) at the study site at the University of Minnesota, Masonic Cancer Center. Only study personnel directly involved with the study will have access to the linked records. The samples that will be transferred to the laboratory or stored for future analyses will be de-identified. We will not be transferring data that has any identifying information over the Internet.

Biomarker samples (blood, urine, saliva and buccal/oral cells) that are banked after the completion of the primary analyses will be stored at the Masonic Cancer Center Hecht laboratory located at the Cancer and Cardiovascular Research Building for future use.

No identifying information will be shared with outside investigators. If used in any collaborative efforts beyond the scope of this study, any shared data will be de-identified. However, records for the study may be reviewed by departments at the University with appropriate regulatory oversight. The records may also be reviewed by a representative of the funding agency, National Institutes of Health, and the Food and Drug Administration.

7.0 Sharing of Results with Participants

Information will not be shared with participants.

8.0 Study Population

Inclusion Criteria:

1. Male or female age 18 years or older. Participants can be smokers or non-smokers;
    a. Smokers must report daily use (at least one cigarette per day)
2. In good physical health with no unstable or serious medical conditions as determined by the licensed medical professional;
3. In stable and good mental health (i.e. not currently, within the past 6 months, experiencing unstable or untreated psychiatric diagnosis, including substance abuse) as determined by the licensed medical professional;
4. Not using any medications that may affect the Nrf2 pathway;
5. Women who are not pregnant or nursing or planning to become pregnant;
6. Participants have provided written informed consent to participate in the study.

Exclusion Criteria:

1. Significant immune system disorders, respiratory diseases, kidney or liver diseases or any other medical disorders that may affect biomarker data as determined by the licensed medical professional;
2. History of Type I or II diabetes;
3. Vital signs outside of the following range (participants failing for vital signs will be allowed to re-screen once):

   a. Systolic BP < 90 or ≥ 160 mmHg
   b. Diastolic BP < 50 or ≥ 100 mmHg
   c. Heart rate < 45 or ≥ 105 bpm
4. Not willing to abstain from eating cruciferous vegetables during the course of the study;
5. Known allergy or contraindication to watercress or any of the study product components;
6. PKU disease (as some of the flavoring packets contain phenylalanine).

Participants who agree to go through the telephone screening process will be assigned a screening number and taken through a screening questionnaire. Participants who meet the eligibility criteria described above will be invited to participate in a virtual screening visit, where the entire study will be explained in detail, informed consent will be obtained and the screening measures will be completed.

9.0 Vulnerable Populations

No vulnerable populations will be used.

10.0 Local Number of Participants

Three hundred and fifty participants are needed for the study. Anticipating a 15% attrition rate, a total of 400 of participants will be enrolled into the study. We expect about 300 non-smokers and about 100 smokers.

11.0 Local Recruitment Methods

We will recruit smokers and non-smokers using various media sources (flyers, newspaper ads, Craigslist, Facebook, etc.). We will also recruit participants who have previously participated in other research studies through the Tobacco Research Programs and agreed to be contacted for future studies.

For each clinic visit, the participant will be paid $25 for the screening visit. Eligible participants will be paid $10 for transportation costs and $25 for time spent at each visit (12 visits × $35 = $420). A $245 bonus will be provided for completion and compliance to the study procedures (including biochemical validation of watercress consumption by measurement of PEITC-NAC in urine) for a total of $690.

12.0 Withdrawal of Participants

A study participant may be discontinued from the study if investigators determine that this is the best decision in order to protect his/her safety. In the event that a participant either withdraws from the study or the investigators decide to discontinue a participant due to an adverse event (AE) or serious adverse event (SAE), the participant will have appropriate follow-up assessments and if necessary, referrals will be made for medical care. The participant experiencing an AE/SAE will be followed until the problem resolves, stabilizes, or is clearly unrelated to the study product. Any AE that remains open will be reviewed and closed at the last study visit.

For the participant's protection, they will be withdrawn immediately from the study if any of the following occur:

1) Cardiovascular disease (CVD) event: Typically include*s* MI (heart attack), PTCA (angioplasty/stenting), bypass surgery, stroke, or peripheral vascular disease. Less common CVD problems would be new cardiac arrhythmias (e.g., new atrial fibrillation) or new valvular disease (e.g., mitral or aortic regurgitation).
2) DVT/PE (deep vein thrombosis/pulmonary embolism, i.e., blood clots in the venous system).
3) Suicide Attempt: A participant will be withdrawn if he/she attempts suicide at any time during participation in the study.
4) Psychiatric Hospitalization: A participant will be withdrawn if he/she is hospitalized for psychiatric reasons at any time during participation in the study.
5) Pregnancy: If a participant becomes pregnant, she be immediately withdrawn from the study. In addition, the licensed medical professional will follow-up after delivery to ask questions about the health of the baby.

The following will be monitored and can lead to the participant being withdrawn by the Principal Investigators or Medical Monitor:

1) Blood pressure (BP) or heart rate (HR) changes: If any of the following occur post-enrollment:
   1) BP is at or above 160 systolic/100 diastolic; 2) BP is below 90 systolic/50 diastolic and the participant is experiencing symptoms listed on the 'Blood Pressure and Heart Rate Symptom Checklist; 3) HR is at or above 105 bpm; 4) or below 45 bpm and the participant is experiencing symptoms listed on the 'Blood Pressure and Heart Rate Symptom Checklist.
2) Medication changes: If a participant begins taking any of the exclusionary medications or other medications that could potentially have an interaction post-enrollment, the licensed medical professional will determine how best to monitor and minimize potential risks (including withdrawal if warranted).
3) Any hospitalization or debilitation in which participation in the study could be detrimental to the recovery process. This will be self-reported by the participant and reviewed by the investigators and medical professional to determine if continued participation in the study is appropriate.
4) If a participant is behaving in an inappropriate or threatening manner, admits to lying about eligibility criteria, including omitting previous medical diagnoses and medications, is participating in other tobacco research studies that could affect the primary outcome measures, does not follow study instructions, etc., then he/she can be withdrawn at the investigators' discretion.

Participants will be informed about the withdrawal at the visit and data collection will stop. No additional procedures will be conducted if participants are withdrawn. We will ask that all unused product be returned along with completed dosing diaries.

13.0 Risks to Participants

The potential risks for participants recruited for this study are minimal. Physiological and subjective measures will be noninvasive and should present no psychological or medical risk to the subject. Blood and oral samples will be obtained by trained study personnel. Watercress in doses that are administered has the potential to lead to gastrointestinal distress (e.g. flatulence, bloating, nausea). Participants will be under medical supervision throughout their study participation and adverse symptoms will be recorded at each clinic visit and monitored by the project Co-PIs Dr. Hecht and Dr. Hatsukami along with the study's licensed medical professional.

Pregnant women will not be recruited. If participants choose to be sexually active, they should use an appropriate "double barrier" method of birth control (such as female use of a diaphragm, intrauterine device (IUD), or contraceptive sponge, in addition to male use of a condom) or the female should be using prescribed "birth control" pills, injections, or implants. Female participants with child-bearing potential will be tested for pregnancy at the first clinic visit using the first morning void urine sample. If a participant becomes pregnant during the study, she will be withdrawn from the study. Approximately 30 days after being withdrawn or having a positive pregnancy test, the research staff will call the participant to confirm her due date. The licensed medical professional will follow-up with the participant after delivery to ask questions about the baby's health.

14.0 Potential Benefits to Participants

While there might not be any direct benefit to the participant, if proven effective, watercress can become part of a health maintenance diet to possibly reduce risks for certain cancers by enhancing the detoxification of toxicants and carcinogens.

15.0 Statistical Considerations

Descriptive statistics will be used to summarize subject characteristics, for example subject demographics (age and gender, race, ethnicity, income, current occupation and usual occupation), medical history and current health status for current diagnoses, symptoms and past health problems. Categorical variables will be described by their frequencies and the continuous measurements will be described by their mean, median, range, and standard deviations. We will use paired *t*-test to compare distributions of continuous variables (e.g., age, number of cigarettes per day, number of pack-years of cigarettes smoked over lifetime) between the two treatment groups (watercress and placebo arms). The McNemar's Chi-squared test will be used to compare categorical variables, such as sex, *GSTM1 and GSTT1* genotypes between the two treatment groups.

The statistical analysis section particularly focuses on Specific Aim 3, which investigates the hypothesis that detoxification of these noxious agents by conjugation with glutathione, as indicated by mercapturic acid levels in urine, will be significantly elevated compared to placebo in subjects who consumed the watercress preparation and are null for *GSTM1*, *GSTT1*, or both. We can test for a Period x Treatment interaction such as testing if the effect of the watercress preparation varies depending on whether it is given in the first period compared to the second period.

We will have baseline measurements on each subject. Participants will be asked to attend clinic visits on days 0 (baseline), 2, 7 and 14 for each of the periods they are receiving product (watercress or placebo). We will compare the baseline measurements on the outcome variables and some important covariates between the two treatment groups through the two-sample t test to see if there is any significant difference between the two groups in order to ensure homogeneous treatment groups. We will also perform repeated data analysis using the measurements on each subject and consider different baseline parameters for the watercress and the placebo group in our multivariate regression analysis described later.

Measurements: For the comparison between the two treatment groups, we will consider measurements excluding the baseline observation. Measurements will be taken from each subject in the two supplementation sequences as seen in the following diagram:

Group 1: Period #1 (watercress; A1) – washout – Period #2 (placebo; B2)
Group 2: Period #1 (placebo; B1) – washout – Period #2 (watercress A2)

The letter is used to denote supplementation (A for watercress and B for placebo) and the number, 1 or 2, denotes the period. We will assume, in the formulation of statistical models, that the measurements A1, A2, B1, and B2 are "normally distributed" in log-scale. These letters can represent one of our outcome variables: urinary mercapturic acid levels of specific toxicants (e.g. benzene, acrolein, crotonaldehyde, propylene oxide, etc.) or for mercapturic acids generally, or DNA adducts of acrolein.

Main Outcome Variables: Our data analysis will be based on the following "outcome variables":

X1 = B2 – A1; and X2 = B1- A2.

This subtraction will cancel the "within-sequence" effects of all subject-specific factors, leaving only two to be modeled and analyzed: the Treatment (or Supplementation) effect and the Period (or Order) effect. The measurements will result in two independent samples. Each will have the same sample size if there are no dropouts or missing data. Otherwise the number of subjects for each group of measurements (i.e., X1 and X2) will differ if there are dropouts and missing data.

We would obtain values of X1 or X2, which is a log of "ratio" for each subject in each of the two groups with different supplementation order, it's (watercress/placebo) for group 1 and (placebo/watercress) for group 2. We'll then compute the averages of these log ratios, i.e $\overline{\overline{x}}_1$ and $\overline{x}_2$. We will do this calculation separately for the double null group ($\hat{\alpha}_{nn}$), and single null group ($\hat{\alpha}_{np}$). We will compare this average ratio with the average ratio of the double positive group ($\hat{\alpha}_{pp}$). With the double nulls being about 15% of the population, *GSTM1* single nulls (45%) and *GSTT1* single nulls (23%), we expect to see 60 double nulls, 180 *GSTM1* single nulls, 92 *GSTT1* single nulls, and 68 double positive individuals. If there is no influence of *GSTM1* or *GSTT1*, the effect of watercress supplementation would be the same between the double null and the double positive group or between the single null and the double positive group. We will test for the significance of the genotypic effect by using a two-sample t test.

In order to estimate the power for that comparison, we need information on the variability of mercapturic acid levels within and between subjects. We will compute the variances of each of these genotypic groups separately and then obtain a pooled variance estimate to compute our pooled t test statistic. Based on our previous work, we used an intra-subject CV of 0.4 and an inter-subject CV of 0.6, which translates to a pooled standard deviation of 0.657. According to Table 3, the percentage difference between the Placebo and PEITC groups with the double null genotypes varied from 29.8% to 95.4%, whereas the percentage difference for the double positive genotypes varied from 4% to 7%. In general, we will have 80% power to detect a difference of 35% between the double null and double positive group and given the range of difference between these two groups in Table 3, we expect to have good power to study this interaction between watercress and *GSTM1* or *GSTT1* genotypes.

We will also perform the above analysis separately for smokers and non-smokers. We expect to see about 25% smokers (~ 100 smokers) in our sample. We might have limited power to detect the impact of watercress for the double-nulls among the smokers, however this subgroup analysis would reveal if there are suggestive differences in the association of urinary mercapturic acid levels, watercress and *GSTM1*, *GSTT1* between the smokers and the non-smokers.

Multivariate Regression Analysis: We will use multivariate regression analysis to supplement the t-test by pooling the data together and using each of the primary outcome variables as dependent variable, separately. In addition to the variables representing the supplementation, treatment order, and status of *GST (M1, T1)* genotypes, the list of independent variables will include socio-demographic characteristics such as gender, age, smoker vs non-smoker, baseline smoking level (cigarettes per day), and intake of dietary nutrients. We will fit a linear mixed-effect model where the dependency in the repeated measurements on each individual will be captured through a random effect. The status of *GST (M1, T1)* genotypes will be modeled as fixed-effects and we will test for the null hypothesis of no association between the genotypes and each outcome. Our primary parameter of interest will be the interaction between the treatment order (pre vs post) and the genotype groups (double null, *GSTM1* single null, *GSTT1* single null, and double positives). We will perform a t-test to assess the significance of the interaction parameter. The residual covariance structure should be selected among several candidates, i.e. autoregressive, compound symmetry, unstructured, etc., to estimate the fixed and random effects. We do not anticipate much missing data on the participants. In general, we will assume missingness at random (MAR) and the likelihood-based linear mixed effect model will provide consistent estimates of our parameters of interest in presence of MAR. The models will be assessed through their Akaike's Information Criterion and Bayesian's Information Criterion. The normality of the outcome variables will be assessed and in presence of non-normality, we will also implement Generalized Estimating Equations to study the impact of watercress and genotypes on each outcome variable. We will use the statistical software R packages nlm and gee to perform the above analysis. These packages will also provide the flexibility to fit non-linear mixed effect models to assess the impact of watercress and genotype groups.

A variety of measures will be taken to ensure data accuracy and completeness. The regular research team meetings will include discussions of proper methods for data collection, transmission, and storage, limiting data collection to those in protocol required to answer a research question, de-identifying data, and encryption methods.

A comprehensive data dictionary will be created to specify definitions and value codes for all variables that will be entered into REDCap and other study databases. Electronic forms for the collection of subjective measures via REDCap will include programming features to ensure valid data (i.e., input masks, validation criteria, skipout logic) and will be stored on the University of Minnesota, HIPAA compliant, computing system. Double entry will be used for all other de-identified data entered into REDCap. Biological specimens will be labeled with barcode labels that incorporate the participant ID. The secured biospecimen website will identify the location of each sample at the clinic (prior to submission to the laboratory) and in the Hecht laboratory.

Oversight of the randomization and product distribution will be conducted by the Project Manager in collaboration with the co-Principal Investigators. The randomization schedules and the link between the alphabetic code and treatment assignment will be maintained securely by the Project Manager. An Online Randomization and Product Tracking database will be created in collaboration with the study biostatistician. This database will be used to track product purchases, product inventory, and assignment of study product to participants based on the randomization schedule.

Quality control procedures will be conducted for all data collected, including analysis of missing data, and logic checks for out of range and other anomalous values. Queries will be made regarding such data issues, with documentation of any changes made in the data.

16.0 Confidentiality

All investigators and staff associated with this project have been trained, and new hires will be trained, on human research ethics and Good Clinical Practice in accordance with the requirements of the University of Minnesota.

Only the immediate study team (Project Manager, Study Coordinator and Principal Investigators) will have access to individually identifiable private information about participants. Coded ID's will be used throughout the study by all the researchers involved. Original signed consent forms and other identifiable information will be kept separate from the research information in a secure and locked space/database.

17.0 Provisions to Monitor the Data to Ensure the Safety of Participants

Oversight for quality control and adherence to protocol procedures will be conducted by the Project Manager in collaboration with the co-Principal Investigators. A start-up meeting with the whole research team will take place prior to participant enrollment. During this meeting, there will be training on the study protocol, standard operating procedures, equipment and data collection platforms. The co-Principal Investigator, Dorothy Hatsukami, will closely monitor the research staff on the procedures to be used in this study. Such monitoring will consist of frequent in-person discussion of study visits and other procedures to make sure that all protocol procedures are followed, and

regular research team meetings to provide updates on study progress and review the data collection process, the results from data monitoring and other issues of concern.

Standard operating procedures will be developed for consistent implementation of the protocol. The Study Coordinator will be administering all measures during clinic visits and entering the information about each subject into a database. Each visit will have a checklist of all measures that need to be obtained and the order by which they will be administered. The Project Manager will be directly supervising the Study Coordinator and will periodically review protocol compliance and implementation, and adherence to good clinical practice procedures.

The Study Coordinator will go over the questionnaire instructions and will be available to the participant to answer any questions he/she may have. Questionnaires will be reviewed for completeness, however, they may choose not to answer questions.

Once a participant has completed all study procedures and all open events have been closed, the PI will review the participant's binder and sign a form indicating study completion for that participant.

This is a minimal risk, non-therapeutic study. While participating in the trial, AEs and concomitant medications will be assessed at every study visit and vital signs will be obtained periodically. The Co-Principal Investigator, Dorothy Hatsukami will meet regularly with the study staff to review recruitment progress and any adverse events. Entrance criteria will be reviewed following screening. Study participants will be under medical supervision while in the study and our research staff will make appropriate referrals to the physician should any adverse events occur. The Data Safety Monitoring Board (DSMB) and other regulatory bodies will be informed of any adverse events either at the regularly convened meetings or in the annual report, or if necessary, immediately.

The DSMB, potentially comprised of a biostatistician, clinical research scientist (MD) and medicinal chemist, will begin by reviewing the protocol and establishing guidelines for data and safety monitoring. This will include developing standard procedures for day-to-day monitoring by the internal monitors, investigators and study staff. This Board will meet at regular intervals (at least once a year) to evaluate the progress of the trial, review data quality, patient recruitment, study retention, and examine other factors that may affect study outcome. They will also review the participant's ability to achieve the study requirements and the rates of adverse events to determine whether there has been any change in participant risk. Their review will ensure that subject risk does not outweigh the study benefits. A brief report will be generated from each of these meetings for the study record and forwarded to the Institutional Review Boards (IRB).

The DSMB will be available to convene outside of the regular meetings, if necessary, if concerns should arise regarding a particular subject, or any troublesome trends in the subject experiences. They will make appropriate recommendations for changes in protocol, if needed.

AEs will typically be identified during the administration of the Health Changes Questionnaire. Other events may be identified from physiological study measures or by spontaneous reports during assessments.

Assessment of Questionnaire Items

- Health Changes Questionnaire: If the participant answers 'YES' to Questions 1, 2, or 3, the interviewer will assess for an 'Adverse Event.'
    1) *Have you had any negative changes in your health since your last visit?*
    2) *Have you had any changes in medication since your last visit?*
    3) *Since your last visit, have you received any form of medical care?*

Assessment of Physiological Data

- Blood Pressure:
    - The 'Adverse Event Log' and 'Blood Pressure and Heart Rate Symptom Checklist' will be completed if an automatic and subsequent manual blood pressure measurement during the same visit is at or above 160 systolic or 100 diastolic.
    - The 'Adverse Event Log' and 'Blood Pressure and Heart Rate Symptom Checklist' will be completed if an automatic and subsequent manual blood pressure measurement during the same visit is below 90 systolic or 50 diastolic and the participant is experiencing symptoms listed on the 'Blood Pressure and Heart Rate Symptom Checklist.'
- Heart Rate:
    - The 'Adverse Event Log' and 'Blood Pressure and Heart Rate Symptom Checklist' will be completed if an automatic and subsequent manual heart rate measurement during the same visit is at or above 105 bpm.
    - The 'Adverse Event Log' and 'Blood Pressure and Heart Rate Symptom Checklist' will be completed if an automatic and subsequent manual heart rate measurement during the same visit is below 45 bpm and the participant is experiencing symptoms listed on the 'Blood Pressure and Heart Rate Symptom Checklist.'

Adverse Events Communicated by Participants

The occurrence of AEs will be sought by non-directive questioning of the participant at each visit during the study. AEs also may be detected when the participant volunteers them during or between visits or through physical examination, laboratory test, or other assessments.

Review and Reporting of Adverse Events and Serious Adverse Events

Co-Principal Investigators with oversight from Naomi Fujioka, M.D. (Medical Monitor) and the clinic's licensed medical professional will review all AEs and assess whether they are related to the study product.

An AE is defined as the appearance or worsening of any undesirable sign, symptom, or medical condition occurring after starting the study procedures even if the event is not considered to be related to the study product. Medical conditions/diseases present before starting the study are only considered adverse events if they worsen after starting the study product. Abnormal laboratory values or test results constitute adverse events only if they induce clinical signs or symptoms, are considered clinically significant, or require therapy; there are no plans for active monitoring of laboratory tests as part of this project.

To the extent possible, each adverse event will be evaluated to determine:

1. the severity grade (mild, moderate, severe)
2. its relationship to the study product used (suspected/not suspected)
3. its duration (start and end dates or if continuing at final exam)
4. outcome (resolved/improved/unchanged/worsened; study product temporarily interrupted or permanently discontinued due to this adverse event; concomitant medication taken; non-drug therapy administered)
5. whether it constitutes a SAE

Information about all SAEs will be collected and recorded on the project's Serious Adverse Event Report Form. A SAE is defined an undesirable sign, symptom or medical condition which:

1. is fatal or life-threatening;
2. requires or prolongs hospitalization;
3. results in persistent or significant disability/incapacity;
4. constitutes a congenital anomaly or a birth defect;
5. is medically significant, in that it may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

Events not considered to be SAEs are hospitalizations that are:

1. elective or pre-planned, for a pre-existing condition that is unrelated to the products under study and did not worsen;
2. for general care, and/or overnight observation;
3. treatment on an emergency, outpatient basis for an event not fulfilling any of the definitions of serious given above and not resulting in hospital admission.

Safety evaluation will be performed on all accrued participants for whom study products were dispensed. The assessment of safety will be based on the frequency of AEs and severity grade of AEs. Other safety data (e.g. vital signs) will be considered as appropriate.

Any SAE occurring after the participant has signed the consent form and until the last encounter with the participant will be reported. All AEs will be summarized by presenting, for each treatment group, the number and percentage of participants who experienced any AE, the number reporting AEs in each body system and the number of AEs by type. Any other information collected (e.g., severity or relatedness to study product) will be listed as appropriate. A summary of clinically relevant toxic events, such as AEs leading to death or rated as SAEs, those with a suspected relationship to study product, or AEs requiring further medication or non-drug therapies will be provided. Reports will be reviewed regularly by the study investigators.

18.0 Provisions to Protect the Privacy Interests of Participants

It will be made clear to participants that all information obtained during assessments is confidential and that no information will be shared with the participants' clinicians unless the participant requests this in writing.

While all the samples and information will be collected specifically to achieve the goals of this proposal, de-identified individual subject data and back-up samples may be made available to other researchers for research purposes after our study is complete. We will obtain permission from participants to allow de-identified biosamples to be stored in a biorepository for future analyses of biomarkers or genotyping.

There will be no access to medical records or any other sources of private information about the participating participants.

19.0 Compensation for Research-Related Injury

The study poses minimal risk to participants.

20.0 Consent Process

The consenting process will take place virtually via a secured video-conferencing meeting invitation. Potential participants will be told the nature of the research over the phone during pre-screening and then at the screening visit. They will be told they may discontinue participation at any time and will not be discriminated against if they choose to do so. Interested subjects will be provided considerable time to review the consent form, consider whether or not to participate, and have any questions answered by the coordinator. Participants will be required to demonstrate an understanding of the study purpose and procedures prior to signing the consent form. Assessment of the subject's understanding will be completed via questions by a slideshow presentation. The consent form must be signed before the research is started. Immediately after signing, the participant will received an email with a signed copy of the consent form.

The electronic consent forms will be stored in a REDCap database with restricted access for essential study personnel only. The electronic informed consent (eIC) will be built us the 'UMN e-Consent HRP-592-TEMPLATE-Biomedical'. The template will be customized to match the written informed consent form exactly. The electronic signatures obtain in the outline above are intended to be the equivalent of handwritten signatures. Therefore, the electronic signatures will occur in accordance with the predicated rule (e.g. approved, reviewed and verified) as outlined in the Food and Drug Adminstrations's CFR part 11.

21.0 Setting

The study will be conducted at the University of Minnesota, Twin Cities and The Hormel Institute

- Subject recruitment and sample collection will take place at Tobacco Research Programs (717 Delaware St. SE, Minneapolis, MN 55414) and The Hormel Institute 801 16th Ave NE, Austin, MN 55912.
- Biochemical analysis will be carried out in the Masonic Cancer Center (2231 6th St. SE, Minneapolis, MN 55455).

22.0 Multi-Site Research

A total of 400 of participants will be enrolled into the study. Each site will recruit smokers and non-smokers using various media sources (flyers, newspaper ads, Craigslist, Facebook, etc.).

Regular teleconferences will be scheduled between the two sites to discuss the study procedures, recruitment, and any safety concerns.

23.0 Resources Available

The lead site for this study will be the University of Minnesota's Tobacco Research Programs housed at the Delaware Clinical Research Unit at 717 Delaware St. SE Minneapolis, MN. Dr. Dorothy Hatsukami serves as the Director for this Program. We have a Research Project Manager who oversees all research and is responsible for logistics of implementing the protocols and standard operating procedures. She is also responsible for the quality control of the projects by ensuring that all studies follow ethical scientific standards and that procedures meet GCP standards, that all regulatory forms are completed including Institutional Review Board applications, and that the DSMB process is in place. We also have an Administrator who ensures the smooth operation of the daily activities of the Program. In addition, the Program has a registered nurse practitioner and many research project coordinators. The shared space at the Delaware Clinical Research Units includes a shared waiting room with a receptionist, 7 physical exam rooms (two dedicated to the Tobacco Research Programs), 1 phlebotomy room, 5 interview rooms, 2 day hospital rooms, an infusion room, 1 smoking laboratory with one way observation room, laboratory space for processing blood, urine processing laboratory, a locked medication supply room, locked protocol room for subject files, cubicles for data entry, management and analyses, locked supply storage and access to three conference rooms. Two restrooms are in the clinical space for urine collections. We have dedicated space for our biorepository with key card access containing ten -20 freezers. We also have access to all of the resources of the University of Minnesota for our use, as needed.

24.0 References

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