A SWEET CHERRY FEEDING TRIAL IN HEALTHY, OVERWEIGHT MALES: ANTHOCYANIN BIOAVAILABILITY AND INFLAMMATORY BIOMARKER RESPONSE

by

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ABSTRACT

Background: Low-grade chronic inflammation has been implicated as a risk factor in prostate-related pathologies including benign hyperplasia and cancer. Age and obesity are considered proinflammatory states and are established risk factors for PCa. Given the epidemic of obesity as well as the advancing age of the US population, there is a heightened interest in the development of diet-derived bioactive compounds with anti-inflammatory properties. Sweet cherry containing the bioactive anthocyanin (ACN), has demonstrated tumor inhibitory action in model systems, specifically inhibition of inflammatory molecules and prostaglandin biosynthesis. However, the metabolism and ACN doses exposure in cherries remains uncertain. As such, there is interest to establish the exposure levels of cherry derived bioactive in humans to develop a whole food approach to reduce chronic inflammation as a strategy to reduce PCa risk. **Objective:** To assess the urinary and plasma concentrations of ACN from the daily consumption of 3 cups of sweet cherries for 4 weeks and test the relationship of ACN levels and cherry consumption to inflammatory biomarkers in an at risk population. **Design:** This single arm clinical intervention trial enrolled men during the 2011 summer.

Results: Cherry ACN levels differed by significantly by batch resulting in intra and inter-individual variability in ACN exposure. A significant detection of circulating and excreted C3RUT was observed. Prostaglandin E2 Metabolite (PGEM) levels were reduced with cherry consumption in men with elevated baseline values.

Conclusion: We conclude that 1c (142g) of sweet cherries 3 times daily for 4 weeks

significantly reduced the COX-2 metabolite, PGEM, in men with elevated baseline levels. This was the first study to examine the chronic effects of daily sweet cherries on COX-2 inhibition in a population of men at elevated risk for PCa.

CHAPTER 1: INTRODUCTION

Inflammation and Risk of Prostate Cancer.

Prostate cancer (PCa) is the most common cancer among men in the United States, with approximately 223,300 diagnoses and 29,000 PCa related deaths each year [4]. An estimated 30% of men use some complementary approach to their prostate care including vitamin supplements and diet intervention [5]. Low-grade chronic inflammation has been implicated as a risk factor in prostate-related pathologies including benign hyperplasia and cancer [6, 7]. Further, obesity [8] and aging [9] are considered proinflammatory states and are established risk factors for PCa. Given the epidemic of obesity as well as the advancing age of the US population, there is a heightened interest in the development and use of pharmaceuticals and diet-derived bioactive compounds with anti-inflammatory properties [10, 11]. The identification of dietary bioactives with anti-inflammatory properties is an attractive, presumptively safer dietary alternative to prescriptive use of non-steroidal anti-inflammatory agents (NSAIDs) for reducing prostate disease and cancer prevention.

The process of PCa development is a consequence of genetic and epigenetic alterations that transform normal glandular epithelium to preneoplastic lesions with progression to invasive carcinoma [7]. PCa is a predominantly sporadic disease arising in men over the age of 50, with 75-85% of the occurrence in men over 65 years of age [9], reinforcing epidemiological studies which suggest that PCa is a hormone-dependent cancer associated with prostatic inflammation [6]. Results

from observational studies show positive associations of PCa risk with chronic inflammation such as sexually transmitted infections, history of prostatisis, obesity and metabolic syndrome, and inflammatory dietary intake patterns [12]. Clinical evidence also indicates inflammation as an initiator of PCa. The Reduction by Dutasteride of Prostate Cancer Events (REDUCE) trial, examined the effects of inflammation in 5,597 men with elevated prostate specific antigen (PSA) levels. The REDUCE trial found histologic inflammation in >78% of men enrolled in the study, demonstrating inflammation's ubiquitous nature in aging men [13].

The prostate is considered an immunocompetent organ and is populated with leukocytes that increase in number with age, and consist of stromal and intraepithelial lymphocytes (70-80% T cells, 10-15% B cells), macrophages, and monocytes exhibiting strong inflammatory potential [6, 12]. Once activated these cells can secrete various inflammatory cytokines (e.g. IL-6, IL-8, TNF-α)[12]. It is hypothesized that this activation occurs via a combination of bacterial infections, urine reflux, dietary factors, hormones and an autoimmune response that can trigger the inflammatory cascade leading to chronic epithelial injury [6]. Injury to the prostate epithelium elicits a stress and regenerative response, characterized by proliferative inflammatory atrophy (PIA) or proliferative atrophy (PA) lesions [14]. This term was first used by Ruska et al. in 1998 to describe atrophic epithelia that exhibit an increased proliferation index and a reduced apoptotic rate compared to normal epithelium [15]. The frequency of the PIA in the prostate has been investigated, and results indicate that lesions are highly associated with chronic

inflammation seen in PCa [12]. Histological transitions between areas of lesions and PCa, as well as prostate intraepithelial neoplasia (PIN), a precursor to PCa, have been observed [12, 15]. The exact molecular and cellular mechanisms involving stromal and epithelial components of the prostate leading to PCa remains unclear, however, chronic up-regulation of inflammation is consistently observed in PCa patients and is considered a risk factor for the development of PCa, [6, 12] processes that may be accelerated in overweight/obese men [16].

Less than 10% of PCa occurs as the result of a single inherited gene defect, while a much larger fraction of cases arise through multiple genetic and environmental interactions. [7] One of the most commonly reported environmental risk factors is obesity [17], and the associated inflammatory effects of adipokines within adipose tissue [8]. With 1 in 3 men at risk of PCa and 33.8% of adult males demonstrating overweight/obese status [18], PCa represents an ideal cancer for chemoprevention. Independent of age, obesity produces a chronic inflammatory state, potentially leading to PCa. Although studies assessing the association between obesity and PCa incidence have yielded inconsistent results [17, 19], conclusive evidence exists that obesity is associated with PCa aggressiveness, progression and mortality [16]. The principle mechanism that links obesity to PCa is the production of adipokines (e.g. leptin, vascular endothelial growth factor) [8]. These pro-inflammatory effects of adipose released cytokines may alter tumorgenesis via nuclear signaling pathways [16].

Experimental evidence suggests the use of anti-inflammatory drugs (e.g. NSAIDs) may be useful in PCa prevention. Mahmud et al. (n = 12,238) found a 42% reduction in the odds of PCa occurrence in chronic aspirin users, a non-selective cyclooxygenase (COX) inhibitor (CI 0.82-0.99). Whereas, they found a non-significant reduction of 13% in general NSAID users, (OR: 0.87, CI: 0.61-1.24) [20]. Celecoxib, a selective-COX 2 inhibitor, has been assessed in three phase-II randomized clinical trials in patients post radical prostectomy or radiation therapy. All trials utilized PSA as endpoints, resulting in a 20-28% reduction of circulating PSA compared to a placebo group [21-23]. However, one trial was terminated early due to the increased incidence of cardiovascular risk in the treatment arm [22]. Large clinical trials are limited and have produced inconsistent results on the impact of NSAIDs in primary PCa prevention [12]. Given the potential gastrointestinal adverse effects of NSAIDS and the seriously adverse cardiovascular effects of selective COX-2 inhibitors [20] , further investigation of non-pharmaceutical based anti-inflammatory agents is necessary.

A modest delay in clinically symptomatic disease and/or disease progression could significantly affect the quality of life of men by reducing treatment-associated comorbidities that arise in 50% or more men [7]. Therefore a non-pharmaceutical, anti-inflammatory dietary strategy for PCa is an extremely attractive model for risk reduction.

The Cherry as a Biological Active Fruit for Disease Prevention.

Sweet cherry, a fruit belonging to the genus *Prunus* in the *Rosaceae* family, contains several active compounds that have demonstrated tumor inhibitory activity in model systems [24]. The anti-cancer bioactives of cherries include the fruit's abundant sources of fiber, phytonutrients and antioxidants [25]. The health-promoting biological effects, particularly of anthocyanins (ACN), in regards to inflammatory molecules and prostaglandin biosynthesis [26-29] are of particular relevance to PCa prevention. As such, there is interest to establish the necessary exposure levels of cherry derived bioactive in humans in order to demonstrate an effect of these compounds as a whole food approach to reduce chronic inflammation as a strategy to reduce cancer risk [30].

Cherries and other fruits rich in bioactive polyphenols (e.g., raspberries, blueberries) have been widely studied in several models of inflammation including early phase clinical trials for a number of disease pathologies, such as cancer and cardiovascular disease [31-35]. Studies conducted using cell culture and animal model systems have found fairly consistent effects of cherry bioactives, such as cyanidin (Cy) on biomarkers of inflammation [36-39]. These studies are summarized in Table 1 and 2.

Kim et al. examined the anti-inflammatory effects of phenolics on neuronal cells from different sweet and sour cherry varieties, finding a dose dependent protective effect against cell-damaging oxidative stress, citing strong anti-neurodegenerative activity [40]. Tall et al., examined tart cherry extract's impact in a mouse model of inflammation, finding a dose-dependent reduction in hyperalgesia

Table 1. Cy impact on inflammation in cell lines					
Reference	**Cell line**	**ACN**	**Dose**	**Outcome**	**Primary Results**
Hou et al.	LPS activated macrophages (RAW 264)	5 ACN*	75 µM x 30 min	Effect on expression of COX-2	Cy and Delphinidin treated cells: ↓COX-2 mRNA and protein levels
Seeram et al.	Human tumor cell lines (MCF-7 and SF-268)	5 ACN* (Cy from tart cherry)	40 µM x 48 hrs	Inhibitory effect on COX-1 and COX-2 activity	Cy most potent inhibitor ↓ COX -1 activities by 52.2% ↓ COX-2 activities by 74.2%
Munoz-Espada et al.	Prostate CA (LNCaP)	Cy	0.5-1 µM x 24 hrs	Effect on PGE2 and COX-2 levels	↓PGE2 and COX-2 protein levels ↓ mRNA levels of COX-2 and PPARγ
Wang et al.	THP-1 activated macrophages	C3G	100µM x24 hrs	Determine anti-inflammatory mechanism	↓iNOS and PGE2 mRNA and protein levels ↓NF-κB binding activity ↓IκBα phosphorylation
Choi et al.	H_2O_2 activated human diploid fibroblast (WI-38)	Cy	0.5-10.0 µg/ml	Examine the anti-aging effects of Cy	↓NF-κB, COX-2, iNOS expression ↓lipid peroxidation ↑WI-38 cell life span
Acquariva et al.	DNA derived from plasmid (pBR322)	Cy C3G	100 200 200 µmol/L	Effects on DNA cleavage and free radical scavenging capacity	↓DNA cleavage ↓free radical scavenging
Reddy et al.	Human tumor cell lines (AGS, HCT-116, MCF-7, NCI-H460, SF-268)	C3G	12.5-200 µm/ml	Effects on COX activity and tumor cell growth inhibition	↓lipid peroxidation ↓COX-1 and COX-2 expression ↓Ca cell growth
Shih et al.	Human AGS	5 ACN	100-200µM x 24hrs	Effects on cell cycle progression and induction of apoptosis	↓apoptosis in 24 and 48 hrs post treatment ↑p38 kinase expression ↓ERK activity
Chen et al.	Human breast cancer cell lines (HS578T)	C3G P3G**	2 µM x 5 days	Demonstrate anti-cancer effects of ANC	↓ cell growth via G2/M arrest ↓cylin-dependent kinase-1, 2, cylin B1 and cylin E protein levels

* 5 ACN used: Cyanidin, Delphinidin, Pelargonidin, Peonidin, Malvidin. AGS = gastric adenocarcinoma cells, P2G =Peonidin-3-glucoside

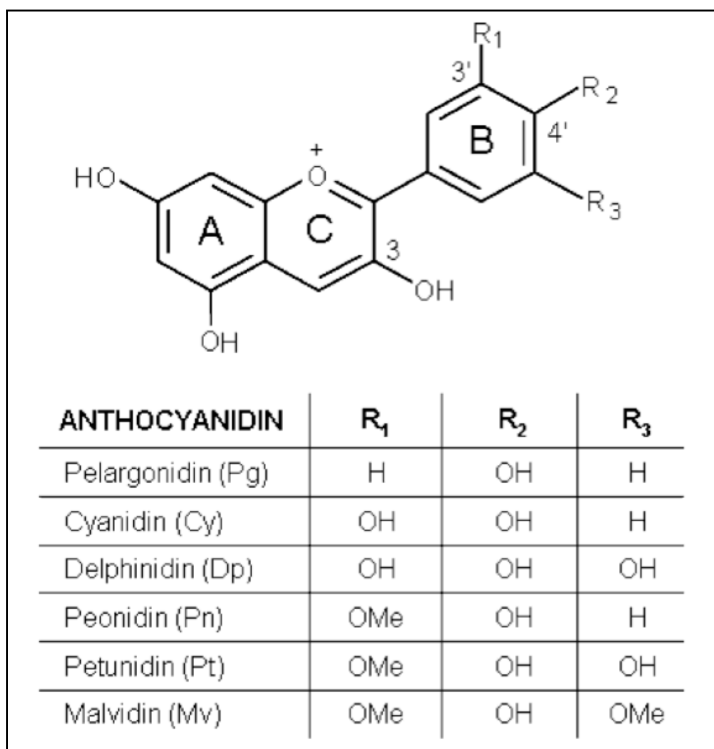
Table 2. Anti-inflammatory effects of cherries in cell lines

Reference	Cell line	ACN	Outcome	Primary Result
Kim et al.	H_2O_2 activated neuron	Sweet and tart cherry phenolic extracts (3-220mg/100g) x 10 min	Impact on neuronal oxidative stress	↑neuronal cell viability ↓oxidative stress
Kang et al.	Human colon cancer (HT 29 and HCT 116)	ANC (0-100 μM) Cy (0-250 μM) x 72 hrs	Inhibition of colon Ca cell growth	↓ cell growth
Anti-inflammatory effects of cherries in animal models				
Reference	**Model**	**Intervention**	**Outcomes**	**Results**
Kang et al.	APC^{Min} mice	Control diet (CD) CD + ANC (800mg/l) CD + Cy (200mg/l) CD + cherry (300g/kg) X 10 weeks	Inhibition of intestinal tumor development	↓frequency and size of cecal adenomas
Tall et al.	Male Sprague Dawley rats*	CD CD + ANC (15mg/kg) CD+ ANC (85mg/kg) CD + ANC (400mg/kg)	Efficacy of orally administered ACN from tart cherries on inflammation-induced pain behavior in rats	↓inflammation-induced thermal hyperalgesia ↓ mechanical hyperalgesia ↓paw edema
He et al.	Male Sprague Dawley rats**	Control diet (CD) CD + ANC (10mg/kg) CD + ANC (20mg/kg) CD + ANC (40mg/kg)	Investigate the anti-inflammatory and anti-oxidative effects	↓TNF-α and PEG2 in seum levels ↑anti-oxidant status
Anti-inflammatory effects of cherries in human models				
Reference	**Participants**	**Intervention**	**Outcomes**	**Results**
Kelley et al.	18 health adults	Bing cherry 280 g/d x 28 days	Determine the effects of sweet cherries on plasma lipids and markers of inflammation	↓CRP, NO and RANTES
Connolly et al.	14 male athletes	24oz tart cherry juice x 8 days	Effects of tart cherry juice in preventing the symptoms of exercise induced muscle damage.	↓self reported pain ↓elbow strength loss
Karlesen et al.	120 healthy adults	Placebo Medox ACN supplement (300mg/day x 3 wks)	ANC supplementation affect on NF-κB activation	↓NF-κB activation ↓IL-8, RANTES, and IFNα

and paw edema [36]. Similarly, He et al., investigated the anti-inflammatory effects of cherries in a rat model of inflammation, finding a significant reduction in the pro-inflammatory mediators tumor necrosis factor-alpha (TNFα) and prostaglandin E2 (PGE2) after 28 days of oral administration of cherry ACN [37]. Additionally, small pilot studies in humans suggest that increased cherry consumption can reduce inflammatory markers in healthy adults. Kelley et al., examined the effects of consuming 300g of Bing sweet cherries for 28 days in 20 health volunteers, and found lowered pooled serum concentrations of potent inflammatory mediators, C-reactive protein (CRP) and nitric oxide (NO) [41]. In a randomized, placebo controlled, crossover trial in 14 male athletes, Connolly et al., found that consuming 12oz of tart cherry juice (estimated to contain 600mg phenolic compounds and 40mg ACN) twice daily for 8 days, significantly reduced self-reported symptoms of exercise induced muscle pain and attenuated measured elbow muscle strength loss following an eccentric exercise program [42]. While a number of questions remain, such as the specific chemical nature producing the anti-inflammatory effects of cherries, the between fruit variation, and the bioavailability in man, collectively, the cell culture, animal model and healthy adult studies are highly suggestive of potent anti-inflammatory activity of cherries.

Anthocyanins (ACNs) as a class of Bioactive Compounds enriched in Cherries

The major class of polyphenols in cherries, hypothesized to have anti-inflammatory and anti-cancer activity, are ACNs [43]. ACNs, water-soluble



ANTHOCYANIDIN	R_1	R_2	R_3
Pelargonidin (Pg)	H	OH	H
Cyanidin (Cy)	OH	OH	H
Delphinidin (Dp)	OH	OH	OH
Peonidin (Pn)	OMe	OH	H
Petunidin (Pt)	OMe	OH	OH
Malvidin (Mv)	OMe	OH	OMe

Figure 1. Six naturally occurring ACN structures (Wu et al. 2002)

flavonoids, are responsible for the brilliant red, purple, or blue color of fruits and vegetables and they are one of the most abundant classes of flavonoids. It is estimated that the average American's daily intake is 180-215mg/day [44]. ACNs are glycosides and aclyglycosides of anthocyanidins, and vary in structure by different hydroxyl or methoxyl substitutions on their flavylium configuration [45].

There are six naturally occurring ACN compounds (see Figure 1). The six ACNs differ by three primary aspects: the number of hydroxyl groups in the molecule or the degree of methylation of these hydroxyl groups, the number and location of the sugars attached to the molecule, or the number and nature of the aromatic acids attached to the sugars in the molecule [2, 44]. Cyanidin (Cy) and its glycosides (CyG) are the major collection of naturally occurring anthocyanins, largely distributed in the human diet [46] and the predominate ACN found in cherries [25]. It is estimated that sweet cherries contain 80mg of ACN per cup, with

approximately 90% attributed to Cy residing in its flesh and skin [47], whereas tart cherries have less ACN as the Cy concentration is limited to the skin [24].

Naturally occurring Cy is typically glycosylated with glucose, galactose or rutinoside at the carbon-3 hydroxyl group [44]. The primary Cy found in sweet cherries is Cy-3-rutinoside (C3RUT), secondary Cy concentration is Cy-3-glucoside (C3G), while minimal amounts of peronidin-3-rutinoside (P2RUT) exist [40, 48]. However, the available data on ACN and polyphenolic content in cherries by species and ripeness is limited and highly inconsistent [49]. Kim et al. evaluated total ACN in sweet and sour cherries using a non-specific colorimetric analysis reporting substantially more phenolic and total ACN in sour cherries compared to sweet cherries. The most ACN-rich sour cherry, the Sumadinka cherry, contains more ACN (109.2mg/100g) compared to the most ACN-rich sweet cherry, the Hartland cherry (76.6mg/100g) [40]. Using high performance liquid chromatography coupled with diode array detector (HPLC-DAD), Serrano et al. examined the total ACN composition of 11 different sweet cherry varietals, finding extreme variability between variety as well as stages of ripening. Brooks cherries were reported to have the lowest concentration of ACN (10mg/100mg), whereas Sonata cherries had the highest (200mg/100mg). Also noted, there was a trend of increasing ACN concentration during the 16 day storage at 2°C post harvest [50]. Determining the ACN pigment content by the pH-differential method via 300-UV spectrophotometer, Chaovanalikit and Wrolstad assessed total ACN concentration and processing effects of 3 varieties of sweet cherries and 1 tart cherry variety. The results revealed Bing, a

sweet dark cherry, to have the highest ACN concentration (60.6mg/100g) where Montmorency tart cherries had the second highest concentration (36.5mg/100g). The pale flesh sweet cherries, Royal Ann and Rainier cherries, had the lowest ACN concentration (2.2mg/100mg and 2.1mg/100mg, respectively) [49]. This extreme variability in the concentration of cherry ACN can be attributed to distinctive ACN measurement methodologies, as well as cherry production and processing conditions [24].

Several pre and post harvest conditions, as well as processing factors, have been described as effecting ACN content in cherries. Pre-harvest factors include temperature, light intensity, fruit crop maturity and soil types. For instance, high temperature and light intensity throughout the growing season can significantly enhance ACN concentration in cherries [25]. Additionally, an increase in harvest stage and fruit ripening can exponentially enhance the total ACN within the same cherry variety [50]. Post-harvest factors such as storage and processing can dramatically decrease cherry ACN concentration as well [25, 49]. Chaovanalikit and Wrolstad found a 75% reduction in ACN content after storage in -20°C for 6 months, compared to a 10% reduction in cherries stored at -80°C for 6 months[49] In 2010, McCune et al., concluded that the total antioxidant activities and exact measurements of ACN in cherries remains unclear [24].

Biological Affects of Specific ACNs.

Cyanidin (Cy), the principal ACN found in cherries, has been reported to attenuate the inflammatory response associated with the initiation of cancer [43, 51, 52]. Proposed mechanisms include inhibition the cyclooxygenase (COX 1 and COX 2) enzymes [28, 53, 54], TNF-α [55], inducible nitric oxide synthase (iNOS) derived nitric oxide (NO) [29], and non-specific inhibition of proliferation [54, 56, 57].

ACNs as natural cyclooxygenase 2 inhibitors.

Cyclooxygenase (COX) catalyzes the initial step in the formation of prostanoids (PG), potent inflammatory mediators implicated in the development of cancer [58, 59]. The COX enzymes consist of at least 2 isoforms, COX-1 and COX-2. COX-1 is expressed ubiquitously in human tissue and plays a role in the production of PG in normal physiological processes, such as platelet aggregation and gastric acid regulation. COX-2 expression is induced in response to inflammatory and mitogenic stimuli [51]. A large body of evidence indicates COX-2 expression and prostaglandin E2 expression in a number of cancer cell lines, including PCa [51, 58]. Overexpression of COX-2 can increase cell proliferation and inhibit apoptosis influencing both the initiation and progression of cancer [60].

Through peroxidation, COX-2 donates two oxygen molecules to arachidonic acid, which then undergoes reduction to form prostaglandin H2 (PGH2). PGH2 can be synthesized into PGE2 via PGE synthase or into thromboxane A2 (TBXA2) via thromboxane synthase. Both, PGE2 and TBXA2 are mediators in the chronic

inflammatory process leading to tumorigenesis [39, 59, 61]. Epidemiological evidence indicates a significant association between regular NSAID use and a reduction in risk for cancers of various organ sites (breast, colon and prostate) [61]. Over the past two decades, pharmacological inhibition of COX-2 has become a major area of research for targeted cancer prevention [58, 59]. However, as discussed above, the non-selectivity of multiple NSAIDs has multiple associated toxicities including GI effects, increased risk of bleeding, as well as cardiac complications. As a result, alternative strategies have been sought [61]. Accumulating preclinical evidence indicates that the ACN compound Cy, has potent cyclooxygenase inhibitor activity with limited toxicity and, therefore, may offer a less toxic option for prevention [39].

A number of published reports demonstrate Cy's abilities to inhibit the expression of COX-2 in cell lines [54, 62, 63]. Hou et al., examined the effects of five different ACNs on LPS-activated macrophage cells. They found, two ACNs, delphinidin and Cy, significantly inhibited COX-2 expression [39]. In 2001, Seeram et al., found that cherry and raspberry ACN's (125 μM concentration) had the highest reduction in COX inhibition compared to blackberry, blueberry, strawberry, and cranberry. In fact, sweet cherry had the highest COX-2 inhibition at 47.2% and was comparable to the observed COX-2 inhibition of the NSAIDs, ibuprofen and naproxen (10 μM concentrations) [28]. Later, Seeram et al. examined specifically Cy's ability to inhibit COX in human cancer cells, and found a reduction of 52.2% and 74.2% of COX-1 and COX-2 expression, respectively [54]. Specific to PCa cells,

Munoz-Espada et al. treated cells with 1 μM of Cy and found an attenuation of arachidonic acids effect on increasing PGE2, as well as a significant overall reduction of PGE2 production and COX-2 expression, within this cell line [27]. The cell culture evidence supports the study of Cy in cancer prevention as a non-selective COX inhibitor, with a preferential inhibition of COX-2.

The principal mechanism proposed to explain Cy's inhibitory affect on COX-activation is suppression of the functional activation of NF-κB. Multiple studies support Cy activity to reduce NF-κB nuclear translocation via inhibition of IκBα phosphorylation, thus preventing the expression of COX2 and production of inflammatory cytokines and chemokines [62, 64]. The inhibition of IκBα activation occurs through the down-regulation of mitogen-activated protein kinase (MAPK) pathways attributed to Cy antioxidant properties. However, it is has also been proposed that Cy may inhibit transcription of NF-κB by directly inhibiting transcription via up-regulation of the nuclear receptor signaling pathway, liver X receptor α (LXRα). Wang et al., found a dose dependent decrease in PGE2, and proposed NF-κB inhibition due to a significant decrease in IκBα phosphorylation as well as inducing transcription activities of LXRα [29]. A study examining 120 healthy adults, found supplementation with 300mg/day of ACN for 3 weeks mediated several NF-κB related inflammatory mediators, such as IL-8, normal T cell expressed and secreted (RANTES) and IFNα [64].

Cy as ROS scavenger attenuating cyclooxygenase 2 expression.

Cy also serves as a potent antioxidant, scavenging free radicals and protecting against DNA cleavage and lipid peroxidation [26, 53, 65]. Marked elevation of ROS can activate NF-κB translocation to the nucleus, thus inducing COX-2 expression. The antioxidant effects of Cy in sweet cherries could attenuate COX-2 expression, thus potentially decreasing initiation of PCa [51]. These biological properties observed *in vitro* support multiple effects of Cy resulting in the inhibition of known mediators of tumor development [26, 28].

Hypoxia, another component of chronic inflammation present in PCa patients, induces high levels of ROS [6]. ROS compounds, including hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-), hydroperoxyl radical (-OH) and superoxide anion (O_2), promote mutagenic DNA damage, lipid peroxidation and activation of COX-2 expression, which can lead to cancer initiation [66]. Multiple studies have illustrated several antioxidant enzymes, such as superoxide dismutase and glutathione-S-transferase are down regulated in PCa. [67-69]. Cy has a dose-dependent antioxidant potential, directly scavenging free radicals, which are implicated in the initiation of PCa [70]. Choi et al. noted Cy markedly blocked intracellular ROS regeneration, specifically O_2^-, H_2O_2, $ONOO^-$ and NO in cells treated with Cy (0.5-10.0 μg/ml) for 24hours [62]. Acquavivia et al., found Cy and C3G (5-30 μmol/L) prevented DNA damage, as well as dose dependent free radical scavenging activity [65]. Using fluorescence spectroscopy, Mulabagal found a 70% reduction in lipid peroxidation with addition of 250 μg/mL cherry fruit extract [26].

Direct anti-carcinogenic effects of ACN

As well as inhibition of carcinogenic signaling pathways, several studies have suggested Cy can directly inhibit tumor cell growth and induction. Reddy et al., evaluated the effects of multiple soluble natural food colors, including ACN, on COX enzymes and tumor cell growth inhibition in several cancer cell lines. They found all ACN pigments inhibited COX-1 and COX-2 and illustrated a dose-dependent growth inhibition against breast, colon, stomach, central nervous system and lung tumor cells [53]. Shih et al., examined the anti-tumor effects of five aglycone ACN that consisted of Cy and four glycosylated ACN, including C3GLU, in human gastric adenocarcinoma cells and found that ACN inhibited cell growth via induction of apoptosis [57]. Similarly, other studies have found that treatment with C3G, the principle Cy in sweet cherries, resulted in a strong inhibitory effect on tumor cell growth [56]. Examining the effects of ACN in tart cherries, Kang et al., found a significant inhibition of tumor development in the Apc^{min} mouse model of colorectal cancer as well as attenuation of proliferation of human colon cancer cells [71]. This growing evidence implicates the use of Cy in a cancer prevention model.

This extensive preliminary *ex vivo* and *in vitro* evidence suggest Cy may have a potential role as a cancer chemopreventive compound. [29, 56, 57, 72]. However, despite the strong preclinical evidence, ACN metabolism, distribution and bioavailability from dietary sources in man remain poorly understood and controversial [44, 46].

Metabolism and Biodistribution of ACNs from whole food sources

In order for bioactive compounds in cherries to have relevant bioactivity for chemoprevention in humans, bioavailability of these compounds from whole food sources must be assessed. Initially, it was proposed that only aglycones of Cy were absorbed via entrocytes, whereas glycosylated forms were poorly absorbed due to no known specific enzyme that selectively hydrolyze glycosidic bonds [44, 45]. Extremely low levels of circulating ACN plasma levels and urine excretion rates post intake reinforced this theory. For example, only 0.018-0.37% total ACN ingested are thought to be excreted in urine, and plasma levels are typically lower or nondetectable [44]. Detection is complicated by the complex absorption of Cy, which until recently, had not been well established. Furthermore, typical detection methods hinder the accuracy of determining Cy bioavailability as they involve acidification of biomatrix at neutral pH. However, Cy can rapidly be oxidized or reduced, given the pH [46].

Recently, the metabolism of Cy has been more clearly defined for man. It is now believed that Cy is primarily absorbed in the stomach, presumably by the organic anion carrier, bilitranslocase [73] or absorbed by simple diffusion in the jejunum. Wherease, CyG absorption is mediated via sodium dependent glucose transport receptors in the intestinal epithelium [44]. CyG may have several distinct fates in the intestine: pass unchanged into the plasma, undergo methylation on the 3’ or 4’ hydroxyl position, glucuronidation, or hydrolysis by β-glucosidase to Cy. However, due to the plasma pH, the algycone Cy rapidly converts to its end

metabolites phloroglucinol aldehyde and protocatechuic acid (PCA). New detection techniques with greater sensitivity and specificity, as discussed below, have made it possible to capture unchanged glycosylated anthocyanins as well as methylated derivatives and glucoronide conjugates previously not detected in the urine or plasma post ingestion of Cy sources [46, 52, 73]. Notably, once Cy reaches the lower GI tract, low molecular weight catabolites of ACN are absorbed after biotransformation by the colon microflora. These metabolites (benzoic acids and PCA) escape most common detection methods, further obscuring estimated exposure levels of circulating Cy post ingestion [52, 73]. CyG may undergo methylation, glycuronidation or hydrolysis in the intestine, liver, kidney, or tissues to form methylated derivatives, glucuronides conjugates or algycone Cy. It is hypothesized that CyG is methylated via catechol-O-methyltrasferase, or acts as a substrate for UDP-glucose dehydrogenase to form glycoside glucuronides [52]. In a 12-hour pharmakinetic study, Felgines et al.. identified C3G metabolites via HPLC. They found native C3G as well as methylated glycosides, glucuronides of ACN and a sulfoconjugate of Cy in the urine of 5 healthy volunteers post ingestion of 200g of blackberries [74]. Other studies examining the absorption and metabolites of dietary ACNs have found C3G and C3SAM, as well as four other metabolites [45]. The presumed absorption pathway for cyanidin follows the first order of kinetics, as the urinary excretion rate is rapid 3-4 hours post ingestion and subsequently declines [44]. The complicated metabolism of ACN has made it very difficult to accurately

calculate ACNs and metabolite exposure in an individual post ingestion of whole food products, such as cherries.

Conventionally, ACNs are measured and quantified using high-performance liquid chromatography (HPLC) coupled with ultraviolet/visible (UV-vis) spectrophotometry [40, 50]. However, HPLC in general has limited sensitivity, particularly at quantifying very low concentrations of metabolites and conjugates. Furthermore, HPLC-UV requires a long run time to achieve optimal resolution to avoid interference substances. Recently, HPLC coupled with tandem mass spectrometry (LC-MS/MS) has become the preferred technique for rapid detection quantification of small molecules. [75].

Possible Toxicity of ACN

In addition to health-promoting bioactivity, naturally occurring compounds in whole foods may result in toxicity when consumed in large quantities. This potentially includes ACN found in cherries. As stated previously, Cy may be transported to the liver were it can be converted to 3'O-methylated forms (C2SAM), where S-adenosylmethionine would serve as the methyl donor and S-adenosylhomocyteine might be produced as a byproduct (see figure 2) [3, 44]. Epidemiologic studies over the past 30 years have provided ample support for the association of mild hyperhomocysteinemia with an elevated risk of atherothrombosis [76]. Yet several large, prospective trials initiated over the past

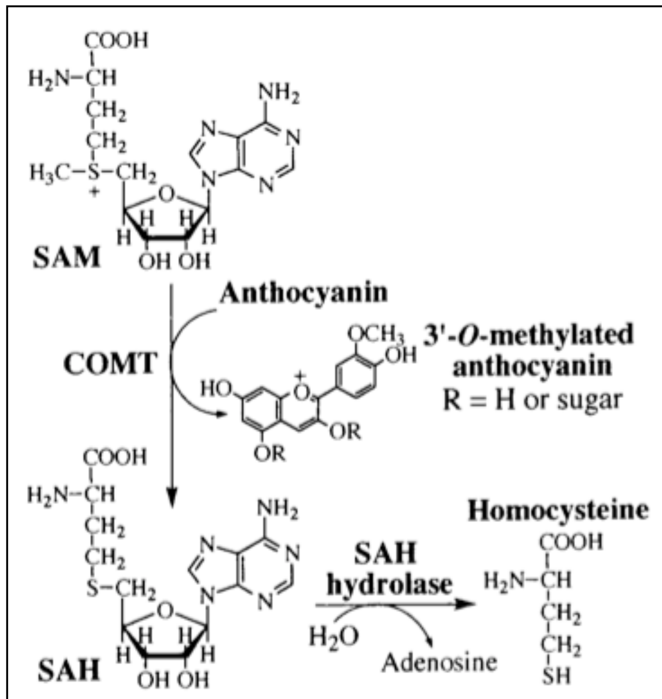


Figure 2. Proposed pathway for the methylation of ACN and production of homocysteine (Nakagawa et al. 2002)

10 years have yet to find an association between lowering serum homocysteine concentrations and decreased rates of vascular events [77] [78, 79]

In a 2002 study, rats were administered a single oral dose of 100mg ACN (50mg C3G, 48mg C3SAM and 2mg Cy-3,5-diglucoside), homocysteine levels were then measured. Mean homocysteine began to rise 90 minutes after ingestion (basal level of 3.7 μmol/L) and continued until the end of the 240 minute experiment, with a final 1.8-fold increase (final mean level of 6.7 μmol/L) [3]. To note, this study used 100mg/100g body weight, substantiality higher dose than typical humans intake (180-215 mg/day). To our knowledge, this has been the only reported toxicity with the use of large doses of ACN.

Consumption of ACN rich food sources, such as cherries, offers a non-drug based approach to deliver these compounds in the diet to attenuate inflammation without the potential of toxicity associated with large dose-effects of pharmaceutical agents. [41, 64].

Current Study

Considering the current gaps in knowledge regarding the bioavailability and effective dose of ACN biological affect in an at risk population, the purpose of this study was to assess the urinary and plasma concentrations of ACN from the daily consumption of 3 cups of sweet cherries for 4 weeks. Additionally, we wished to test the relationship of ACN levels and cherry consumption to inflammatory biomarkers in a population at risk for developing PCa. The first objective was to accurately quantify the exposure of ACN within the 4-week study intervention using the change in plasma and urinary Cy (Specific Aim 1). The structures of the investigated Cy substrates can be seen in figure 3. The second objective was to assess plasma levels of ACNs to determine biodistribution in circulation (Specific Aim 2). The third objective of the study was to evaluate the effect of daily consumption of cherries on urinary and plasma biomarkers of COX-1 (TBX2) and COX-2 (PGEM and CRP) associated activities (Specific Aim 3). The fourth objective was to assess potential toxicity of high cherry

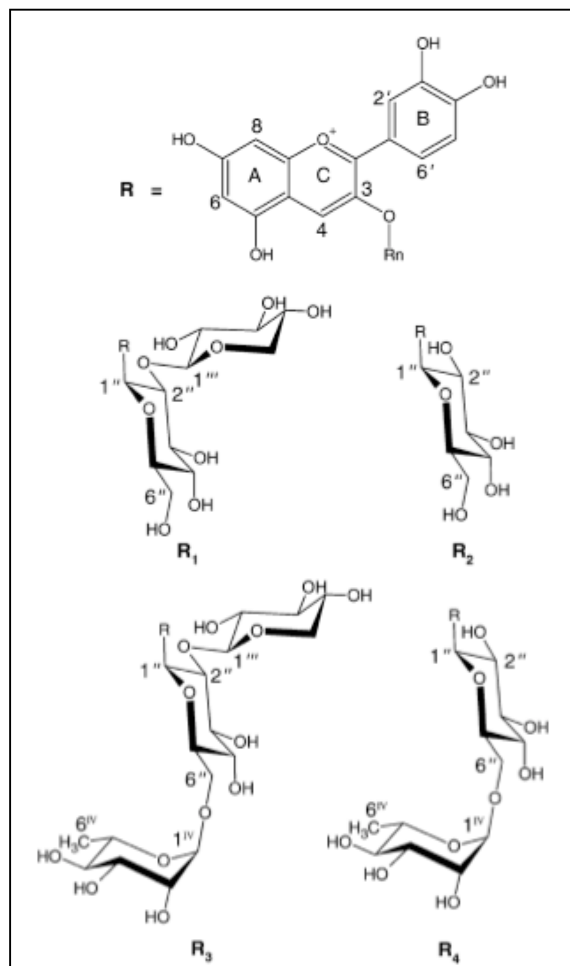


Figure 3. Basic structure of Cy and its glycosides: R1 sambuioside, R2 glucoside, R3 rutinoside, R4 xylosyl-rutinoside (Tulio et al. 2008)

consumption as determined by increased homocysteine levels in man (Specific Aim 3).

CHAPTER 2: METHODS

THE ROLE OF FRESH SWEET CHERRIES IN MODULATING BIOMARKERS OF INFLAMMATION AMONG MALES AT RISK FOR PROSTATE CANCER

Study Sample

One hundred and five overweight and obese men who responded to print, television and electronic advertising were screened via telephone. Eligibility criteria included: men over the age of 50 years, elevated body mass index (BMI) defined as ≥25-45kg/m^2, abstinence from tobacco products (>5 years), discontinuation of dietary supplements (with the exception of a multivitamin for 1 week prior to enrollment and during trial), discontinuation of anti-inflammatory medication (with the exception of a low dose aspirin), absence of co-morbidities (diagnosis of diabetes, cardiovascular disease, inflammatory disease, liver or kidney disease) and cancer-free (> 12 months)

Upon consent, participants began a one-week washout in which they were instructed to avoid foods rich in anthocyanins and to limit consumption of all other fruits and vegetables to five servings per day. The participants completed the study during May 2011-August 2011. Written informed consent was obtained from all study participants prior to enrollment. The University of Arizona Institutional Review Board approved this study protocol.

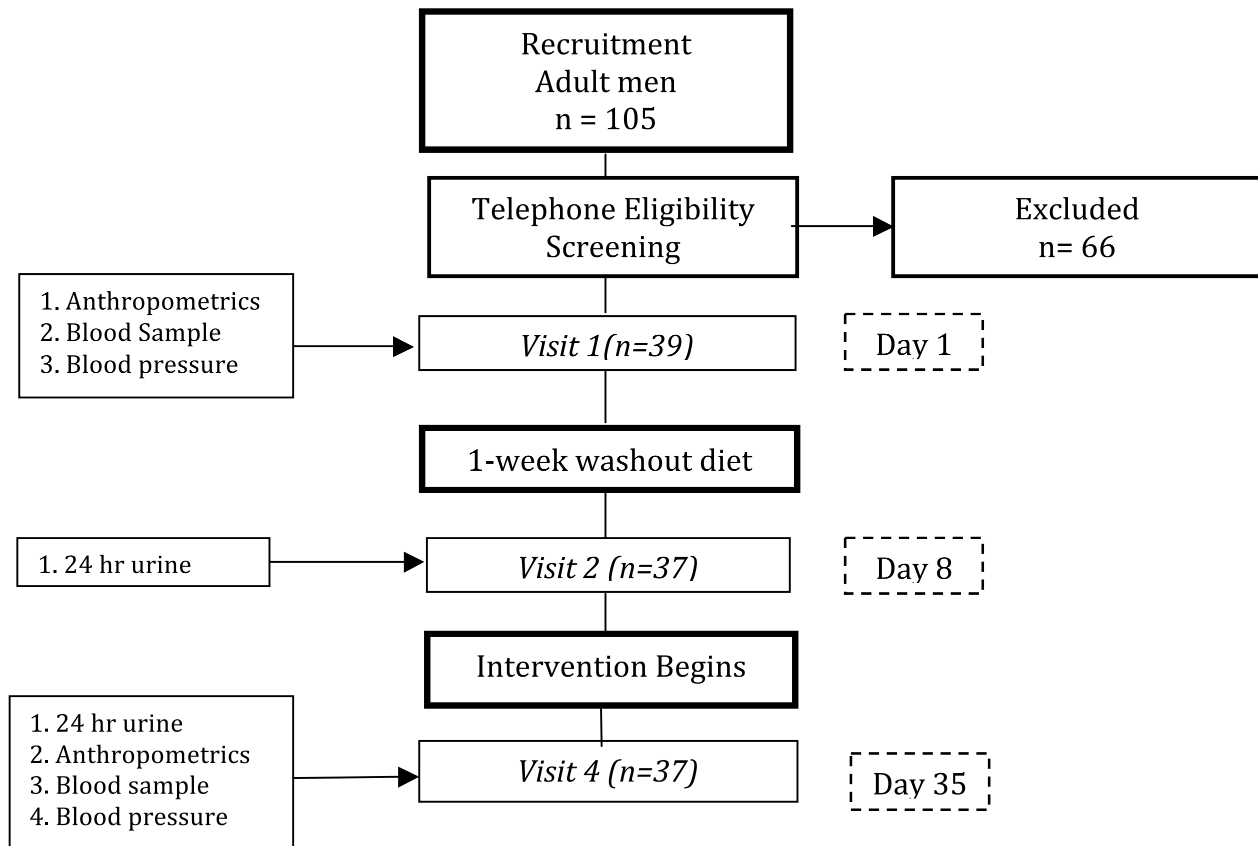
Study Design

This single arm clinical intervention trial enrolled men during the 2011 summer in seasonal correspondence to the U.S. Northwest sweet cherry crop

production. The study consisted of two visits to the clinical research setting at baseline (week 0) and at the end of the 4-week intervention phase (week 5), as well as weekly visits for fresh cherry product. All subjects completed a 24-hour urine collection at the end of the washout period (week 1) and at the conclusion of the intervention (week 5). Fasting blood samples were collected at baseline (week 0) and at the conclusion of the study (week 5). Participants were asked to consume 1 servings of cherries (142g) 1 to 2 hours prior to their final blood draw. Anthropometry and blood pressure measurements were preformed at baseline and the end of study. See Figure 4 for a summary of participant characteristics, timeline, and study activities.

Diet Intervention

Figure 4. CONSORT Diagram



Subjects were advised to maintain body weight, regular dietary intake (other than the addition of study cherries) and physical activity levels throughout the study. Following the baseline visit, subjects entered the washout phase of the study, which limited consumption of fruit and vegetables to 5 servings per day and avoidance of all foods rich in anthocyanins. Subjects were required to discontinue all self-prescribed dietary supplements during the study period, with the exception of a multivitamin. Subjects who successfully completed the washout activities began the intervention portion of the study by consuming 3 cups (426 g) of cherries daily for 4 weeks. Participants were instructed to consume 1 cup of cherries in the morning, afternoon, and evening to reduce any potential gastro-intestinal distress.

Subjects were provided with cherry storage and consumption instructions, as well as a weekly cherry consumption log.

Cherry Processing

Northwest cherry growers in Oregon, Washington, and California comprising the Oregon Sweet Cherry Commission and the Washington State Fruit Commission supplied the sweet cherries. Cherries were delivered in weekly shipments to a local supermarket in Tucson AZ. Upon arrival, the cherries were transferred to The University of Arizona's Nutritional Science Department for processing. The cherries were weighed and separated into daily servings of 426 g per day (142 g per serving) and were assembled into 7-day packages for each subject. Cherries were stored at 9° C until participant pick-up. Subjects picked up cherries weekly at the clinical research facility and were instructed to return the uneaten cherries, pits, and stems the following week. Each participant was provided with a study cooler and two 17.9x17.2 cm ice packs, to ensure proper temperature for cherry transportation. Pits and stems were collected each week to obtain a weekly waste weight.

Outcome Measurements

Diet Composition

Dietary intake was assessed using the previously validated Arizona Food Frequency Questionnaire (AFFQ)[80]. Compliance to the cherry intervention was measured by calculated total cherry weight consumption each week, as well as self-report cherry intake logs. Consumption weight was calculated using the following

formula: [cherry pick-up weight (gm)– cherry return waste (gm)]. Cherry logs were collected at the end of study to ensure diet modification.

Specimen Collection and Processing

Blood samples were collected from the subject's antecubital vein 1-2 hours following consumption. Plasma was collected into a tube with EDTA clot activator, gently inverted and stored at room temperature for 45 minutes to allow for clotting. Both plasma and serum samples were centrifuged at 3,000 rpm for 10 minutes at 4°C in a Sorvall RT 6000B Centrifuge. Following centrifugation samples were aliquot into storage cryovials and stored at -80° C. until analysis.

Twenty-four hour urine was collected using standardized protocols and was stored on ice or under refrigeration throughout the collection period. Upon receipt of urine sample at the research clinic, the collection was measured for total volume. The total collection then was vortexed and two 9ml samples were obtained. The samples were centrifuged at 3,000 rpm for 5 minutes at 4°C in a Sorvall RT 6000B Centrifuge. The centrifuged urine was aliquoted into 2ml urine samples and stored at -80° C until analysis.

Specimen Anthocyanin Measurement

Thirty participants were included in the final plasma and urine ACN analysis. Seven men were excluded from the analysis, as their final consumption of cherries was greater than 4 hours prior to their final visit. EDTA blood samples were collected as described above; plasma was removed and 0.5ml aliquots were diluted with formic acid (FA) to final concentration of 5%. Following acidification, samples

were stored at -80° C until analysis. Calibration standards were acidified with 5% FA to be consistent with the samples. Samples and standards were analyzed using an Agilent 1100 high performance liquid chromatograph interfaced through an atmospheric pressure ion source to a Waters Micromass Quattro Micro tandem quadruple mass spectrometer (LC-MS/MS), at Ohio State University (OSU). Both standards and samples were protein crushed by 1 mL acetonitrile. After thorough mixing by vortex, protein precipitation was further carried on under -20°C for about 5 minutes. The supernatant was separated by centrifugation, transferred to glass tubes and dried under N_2 stream. The samples were reconstituted in 100 μL 25% ACN and 0.1% FA and further cleaned by 0.45μm microfilter prior to LC-MS analysis. Calibration curves of four anthocyanins were linear from 1 up to 500 ng/mL with regression coefficient exceeding 0.99. Quality controls prepared in triplicates at 5 and 50 ng/mL were inserted between sample run to ensure data quality. Level of dictation was set at 1 ng/mL.

Formic acid (FA) was added to the 2ml aliquoted urine to a final concentration of 5%; the samples were stored at -80° C until analysis. The final analysis occurred at Ohio State University in collaboration with Dr. Liu's Biomedical Mass Spectrometry Lab. Calibration standards were acidified with 5% FA to be consistent with the samples. All standards and samples were processed by solid phase extraction. Oasis HLB cartridges were sequentially pre-conditioned and washed after urine samples were loaded. In final step, anthocyanins were eluted by MeOH and 0.1% FA. The eluted fraction was dried under N_2 stream and then the

residue was reconstituted in 100µL 25% ACN and 0.1% FA. Calibration curves of four anthocyanins were linear from 1 up to 1000 ng/mL with regression coefficient exceeding 0.99. Quality controls prepared in triplicates at concentrations of 5 and 50 ng/mL were inserted throughout the run to ensure data quality. Level of dictation was set at 1 ng/mL.

Phenolic Concentrations in Cherries

100g samples of cherries were batched weekly, stems and pits removed. The samples underwent a snap freeze with nitrogen vapor, and were stored at -80° C until analysis. The 11 batches of cherries were analyzed via LS-MS/MS. Calibration standards for four anthocyanins (C3GLU, C3RUT, C3SAM, C3XRUT) with concentration ranging from 1 to 2000 ng/mL were prepared in 25% ACN and 0.1%FA. The berry samples were prepared in triplicates by diluting 11 fold from 10 mg/mL working solutions. The quality control samples were prepared in triplicate at 50 ng/mL and inserted within the sample run. Level of dictation was set at 1 ng/mL.

Anti-inflammatory biomarkers

PGE2 Metabolite (PGEM). In vivo PGE2 is not chemically stable and undergoes rapid converted to 13,14-dihdro-15-keto metabolite. For this reason, human biosamples contain very little PGE2, thus the measure of the metabolites is necessary to provide reliable estimates of actual PGE2 production [81]. Urinary PGE2 levels were detected using the Prostaglandin E Metabolite ELISA kit from Caymen Chemical (catalog # 514531). Standards and controls were prepared

following the instructions of the manufacturer. The lower and upper limits of detection of this assay were 2 to 11 pg/mL, respectively. All paired samples were run on a single plate. Data were considered valid if the intra-assay CV was <10%.

Thromboxane B2 (TBXB2). Urinary TBXB2 was detected using the Thromboxane B2 Metabolite ELISA kit from R&D Systems (Catalog #KGE011). Standards and controls were prepared following the instructions of the manufacturer. The lower and upper limits of detection of this assay were 0.214 to 20 ng/ml . The quality of performance of this inhibition assay was inferior to the other biomarkers measured, with an overall CV >10%.

High sensitivity C-reactive protein (hsCRP). Serum hsCRP was measured using the High Sensitivity C-Reactive Protein ELISA kit from US Biological (catalog # C790-20H). Standards and controls were prepared following the instructions of the manufacturer. The lower and upper limits of detection for this assay were 0.1 to 10mg/L). All paired samples were run in single plate. Data was considered valid if the intra-assay CV was <10%.

Safety Outcome

Serum levels of homocysteine were measured at baseline and end of study to determine if the study dose of ACN was toxic. Samples were processed at Lab Corp; Tucson, AZ.

Anthropometrics

Anthropometry was preformed at baseline and end of study. Subject height was measured using a wall-mounted stadiometer, without shoes to the nearest

quarter inch. Using a calibrated double ruler standing scale, weight was measured without shoes to the nearest half pound. Waist and hip circumference were measured using a Gulick II measuring tape to the nearest quarter inch. The measuring tape was extended around the waist, using the umbilicus, and the iliac crest as the anatomical points of reference [82]. Body fat was estimated using a handheld Omron Body Fat Analyzer HBF-306 (Omron Healthcare, Inc.; Vernon Hills, IL).

Blood Pressure Measurement

Blood pressure was measured in duplicate by an automatic blood pressure monitor by ReliOn HEM-780REL (Omron, Inc.; Bannockburn, IL). Participants rested at least 15 minutes prior to taking the measurement; feet were place flat on the floor and subjects were asked not to speak during the measurement. Systolic, diastolic, and heart rate in beats per minute were recorded separately and the average of the two measurements were used in the analysis.

Secondary outcome measures

To determine the possible attenuation of pain associated with daily functioning, a general quality of life scale was provided to measure the possible effects of daily consumption of sweet cherries. Quality of life was assessed by the MOS 36-Item Short-Form Survey (SF-36). Eight subscales (physical functioning, role-physical, bodily pain, vitality, general health, social function, role-emotions and mental health) were calculated, per standard scoring protocol. Scores range from 0-100 with a higher score indicating a better state of perceived general health [83].

Bowel habits were tracked using the previously validated bowel habits form, to determine if there were significant alterations in bowel habits during the duration of the intervention period [84].

Statistical Analysis

All data were analyzed using Stata version 12.0 (StataCorp, College Station, TX). Results are presented as mean± standard deviation. The sample size of 30 was considered adequate with 80% power and an alpha of 0.05, based on a two-sided dependent student's t-test for testing alterations in biomarker differences between and pre and post intervention. Detection of circulating ACN was treated as a binary variable and analyzed using McNarmer's test. Change in anthropometric, blood pressure, dietary intake, bowel habits and biomarkers were compared from baseline to post-intervention using paired student's t-tests. SF-36 results were compared at baseline and post intervention using Wilcoxon signed-rank test. Linear regression was used to determine the relationship between PGEM and hsCRP change controlling for baseline values. Differences were considered statistically significant at $p<0.05$ for all tests.

CHAPTER 3: RESULTS

THE ROLE OF FRESH SWEET CHERRIES IN MODULATING BIOMARKERS OF INFLAMMATION AMONG MALES AT RISK FOR PROSTATE CANCER

Baseline Characteristics of Study Population.

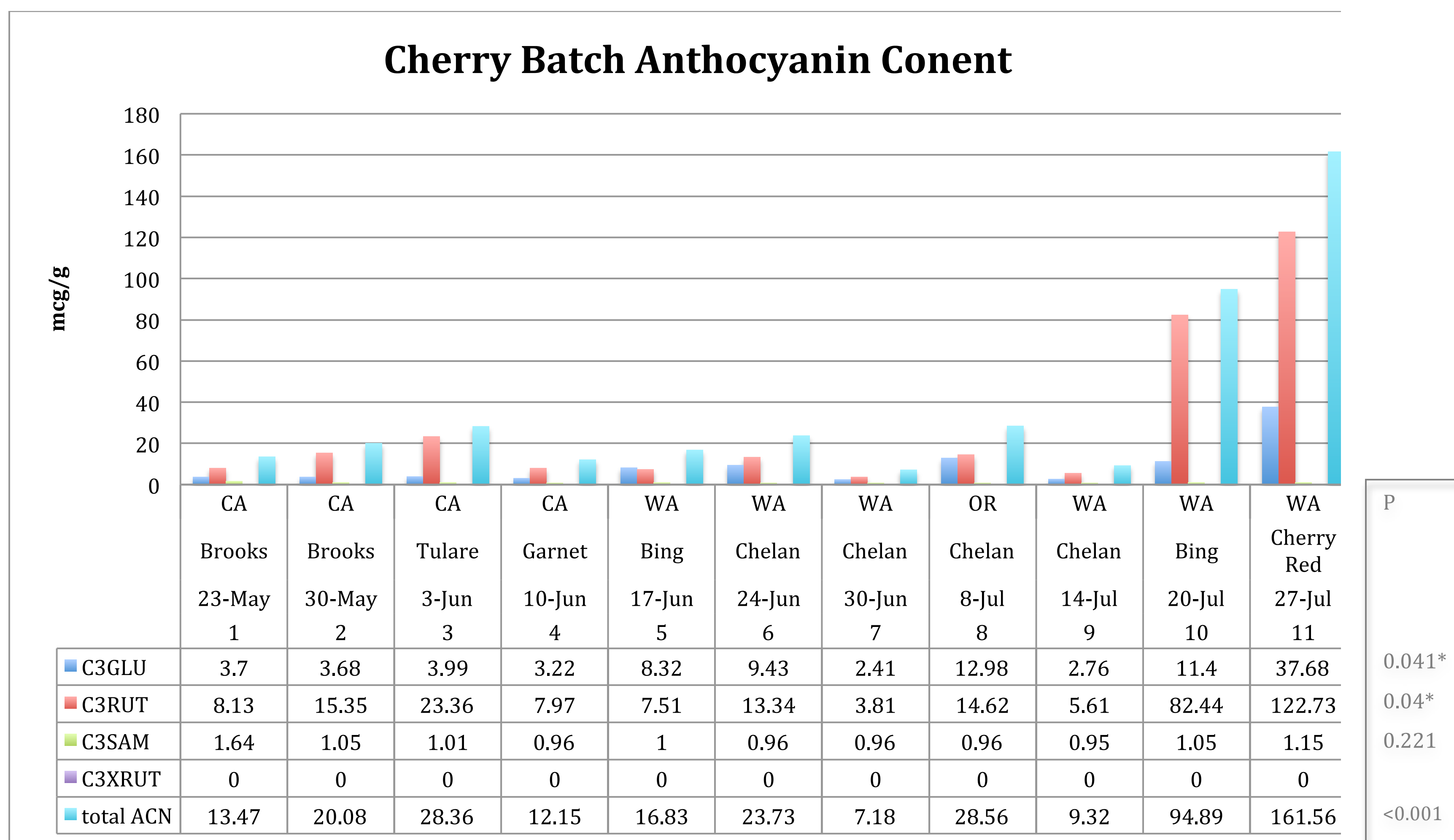
During the 3-month enrollment period, 105 participants were screened and 39 men were identified as eligible and enrolled into the washout phase. The most common exclusion criterion was a history of chronic illness, 12 (11.4%). Other reasons for exclusion included: 9 (8.6%) availability, 9 (8.6%) female, 7 (6.7%) BMI ≤ 25 kg/m^2 and 6 (5.7%) age < 50 years with 'other' defining the remaining 14 (13.3%). Of the 39 eligible men, 37 successfully completed the washout phase of the trial and the cherry feeding trial (see Figure 4). Table 3 summarizes participant baseline characteristics. The study population was between 50 and 83 years of age, with a mean age of 61.1±7.6 years. Of the participants, 4 (10.3%) were Hispanic; the remaining 35 (89.7%) were non-Hispanic white. The population was well educated with 14 (35.9%) having achieved college degrees and another 14 (35.9%) having completed graduate degrees. As per eligibility criteria, all subjects were overweight with a mean weight of 102.5±13.4 kg and an average BMI of 31.5±4.3 kg/m^2. Average blood pressure was considered mild-hypertensive, 133.6±11.0 mmHg systolic and 83.4±9.2 mmHg diastolic.

Cherry ACN levels differed significantly by batch, resulting in intra and inter-individual variability in ACN exposure.

Individual ACN content by cherry variety and batch is shown in Figure 5. As shown, there was significant batch-to-batch variability for total ACN (7.2-161.6 μg/g), C3GLU (2.4-37.7 μg/g) and (C3RUT3.81-122.7 μg/g). C3SAM showed more consistent levels between batches (0.95-1.64 μg/g) and C3XRUT was not detected in any of the batches. There was a significant trend in increased concentration of C3GLU ($p = 0.041$), C3RUT ($p = 0.04$) and total ACN ($p = 0.035$) with the progression of the cherry season. The increasing concentration of the ACNs in the cherries during the study resulted in a significant difference in participant ACN exposure during the timeframe of the study. For instance, the later enrolled participants had a higher exposure to total ACN ($p < 0.001$) and all detectable individual ACNs: C3GLU ($p < 0.001$), C3RUT ($p = 0.029$) and C3SAM ($p = 0.007$), see Figure 5.

Detection of circulating and excreted anthocyanin metabolites

Consistent with their low abundance in the cherries, we observed no statistically significant change in circulating plasma concentrations of C3SAM or C2XRUT (see Table 8). However, we did observe a significant increase in circulating C3RUT ($p < 0.001$) and a significant decrease in plasma C3GLU ($p = 0.006$). Similarly, no statistically significant changes were found in urinary excretion of C3RUT, C3SAM or C3XRUT; however a significant increase in C3RUT excretion ($p < 0.001$) was observed.



	CA Brooks 23-May 1	CA Brooks 30-May 2	CA Tulare 3-Jun 3	CA Garnet 10-Jun 4	WA Bing 17-Jun 5	WA Chelan 24-Jun 6	WA Chelan 30-Jun 7	OR Chelan 8-Jul 8	WA Chelan 14-Jul 9	WA Bing 20-Jul 10	WA Cherry Red 27-Jul 11	P
C3GLU	3.7	3.68	3.99	3.22	8.32	9.43	2.41	12.98	2.76	11.4	37.68	0.041*
C3RUT	8.13	15.35	23.36	7.97	7.51	13.34	3.81	14.62	5.61	82.44	122.73	0.04*
C3SAM	1.64	1.05	1.01	0.96	1	0.96	0.96	0.96	0.95	1.05	1.15	0.221
C3XRUT	0	0	0	0	0	0	0	0	0	0	0	
total ACN	13.47	20.08	28.36	12.15	16.83	23.73	7.18	28.56	9.32	94.89	161.56	<0.001

Figure 5. ACN batch concentration

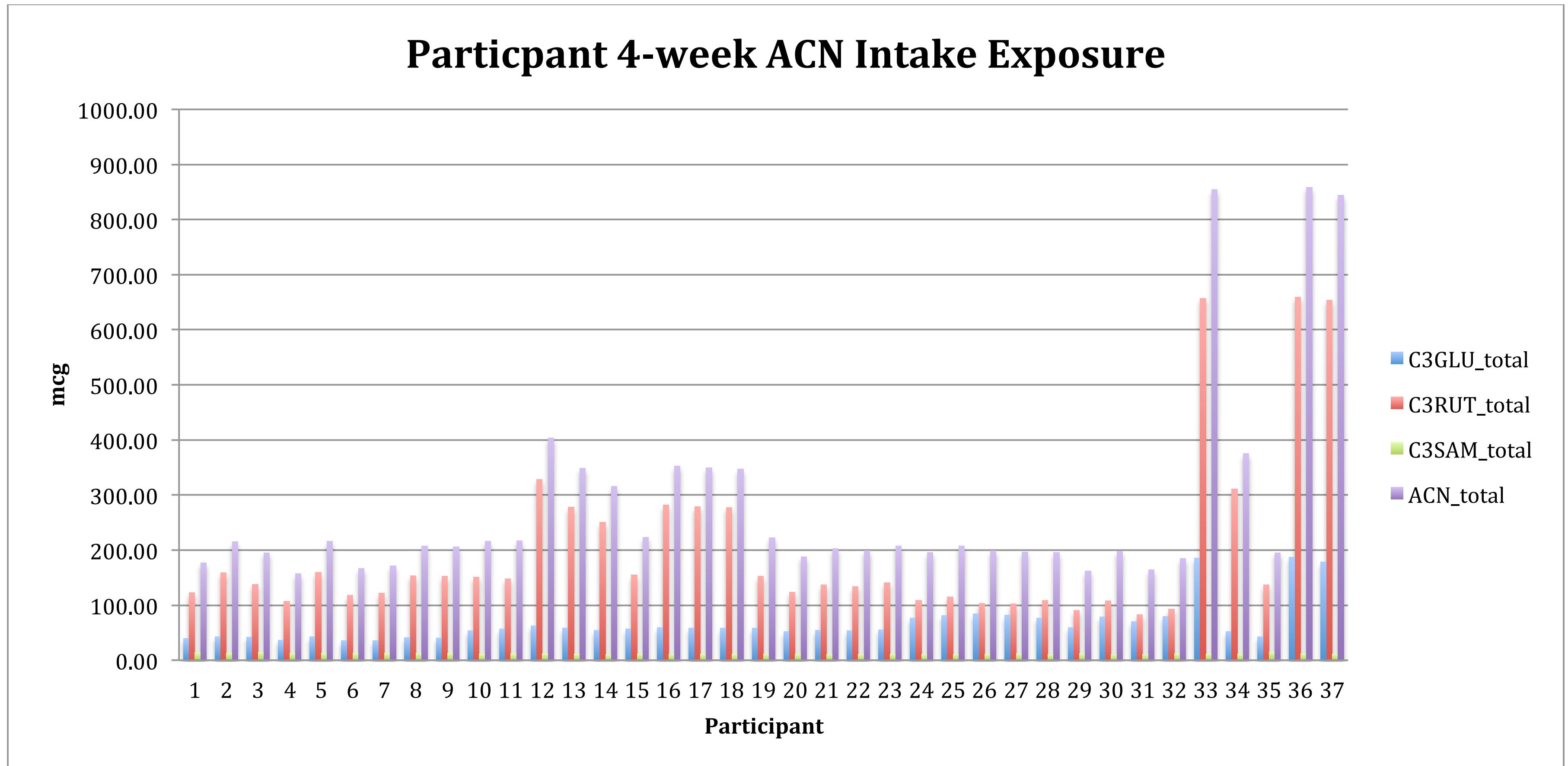


Figure 6. Participant 4-week ACN exposure

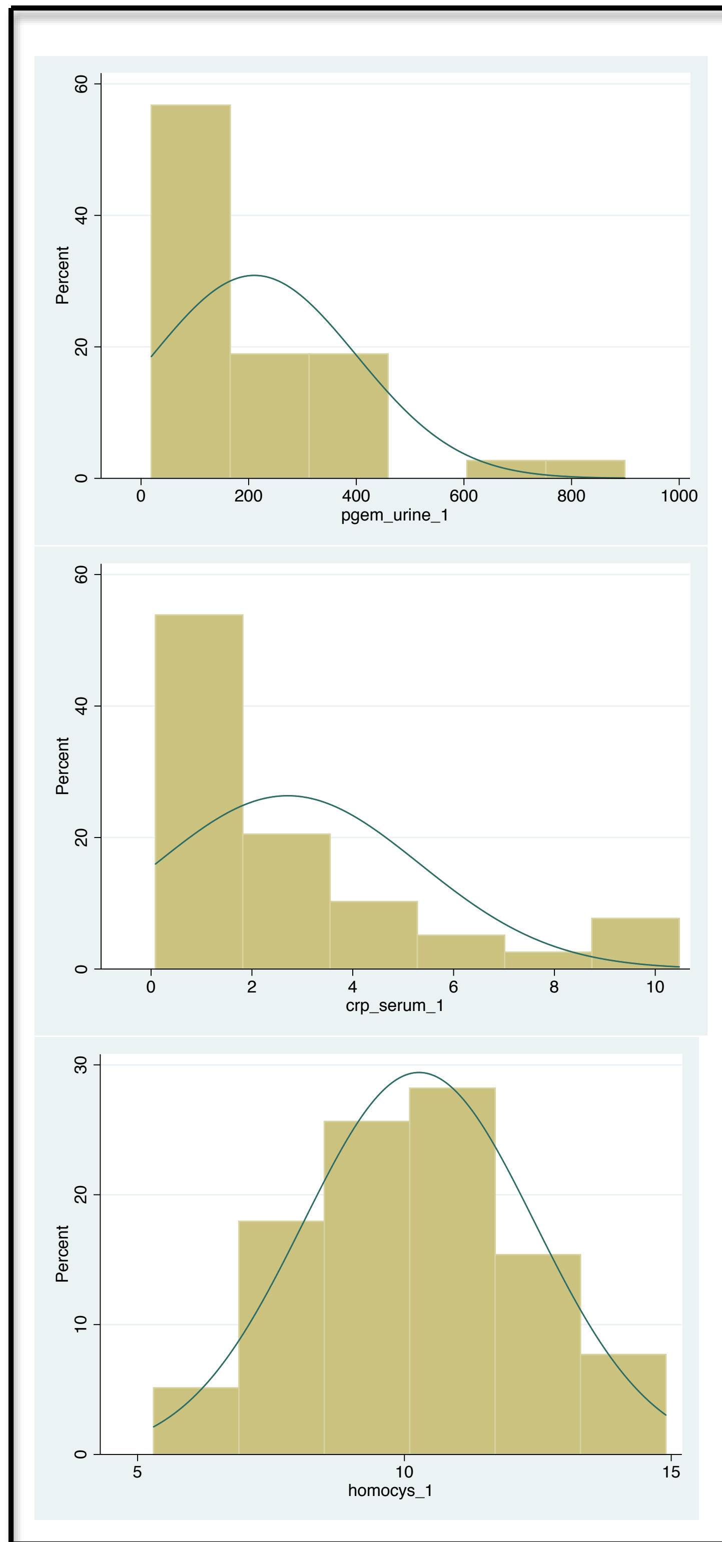


Figure 7. Biomarker baseline distribution. A. PGEM B. CRP C. Homocysteine

Prostaglandin E2 Metabolite (PGEM) levels were reduced with cherry consumption in men with elevated baseline values.

The mean PGEM at baseline was 210.6± 189.5 pg/mL. After the 4-week intervention, we observed a non-significant reduction of 20.9±92.6 pg/mL (p = 0.454) (post intervention mean 189.7±179.4 pg/mL). However, given PGEM's skewed distribution, as seen in Figure 7 (median = 131.4, mean 210.6 pg/mL), the data was log transformed. To explore the relationship between change in PGEM during the intervention period considering baseline values, a linear regression was performed, showing a significant inverse relationship between the change in PGEM and baseline values (-0.25±0.09 β-coefficient and p = 0.009), suggesting an effect by baseline or starting concentrations. Therefore, stratification on the median value (131.4 pg/mL) was performed, and a paired t-test in groups above and below the median was conducted. A significant reduction of -55.5 pg/mL was found in the group with baseline measures above the median (p = 0.014) whereas we observed no effect of cherry consumption on PGEM levels in the men with baseline values below the median (p = 0.473). When consumption was stratified on high or low ANC intake, we found no evidence for a dose effect for reduction of PGEM levels (summarized in Table 7).

The circulating exposure of ACN was compared with PGEM values. Specifically PGEM change was compared with circulating and excreted C3RUT, the only statistically significant increased ACN. No association between change in PGEM

from baseline to end of study was observed in the group with detectable plasma or urinary levels of C3RUT.

Cherry consumption had no effect on urinary thromboxane B2 (TBX2) levels.

There was no evidence of an effect of cherry consumption on TBX2 with presence of high intra-assay variation limiting detection to change > or < 20%. TBX2 analysis was not further pursued due to minimal biomarker effect coupled with poor assay quality leading to possible spurious findings.

Cherry consumption resulted in a significant increase in high sensitivity C-reactive protein (hsCRP) in a population with low baseline values.

The mean hsCRP at baseline was 2.7± 2.7 mg/L. After the 4-week intervention mean hsCRP was 3.0±2.8 mg/L, demonstrating a non-significant increase of 0.3±2.1 mg/L (p = 0.446). However, hsCRP showed a highly skewed distribution in the study population, as seen in figure 7 (median = 1.54 mg/L, mean 2.71 mg/L). Thus, similar to PGE, hsCPR was log transformed to achieve a more normal distribution. To explore the relationship between change in hsCRP during the intervention period and baseline values, a linear regression was performed, which showed a significant inverse relationship between the change in hsCRP and initial values (-0.37±0.1 β-coefficient and p = 0.003). After stratifying on the median value (1.5 mg/L), a paired t-test was preformed in both groups above and below the median. A significant increase of 0.6±0.9 mg/L was observed in the group below the median (p = 0.011), and a non-significant reduction (0.1 mg/L) was found in the

group above the median (p=0.302). After stratifying ACN intake levels into high and low exposure, see Table 7, no significant changes in hsCRP were observed. Circulating exposure of ACN was compared with hsCRP values. Specifically hsCRP change was compared with circulating and excreted C3RUT, the only statistically significant increased ACN. We observed no association between change in hsCRP from baseline to end of study comparing individuals with detectable plasma or urine levels of C3RUT compared to those without detectable levels.

Cherry consumption resulted in minimal but significant alterations in diet composition during the intervention.

Energy intake, macronutrients and micronutrients content of the diets reported via FFQ are summarized in Table 4. Diet composition alterations were observed between baseline and end of study. Mean macronutrients, including carbohydrates, fat, saturated fat and kcals, remained consistent during the 5-week study with the exception of protein, baseline average value of 92.9 ±49.5 to 77.2±31.9 g/day (p = 0.04) at the end of study. Also, a decrease in folate from 491.7±295.2 to 404.7± 174.7 μg/day (p = 0.48) and in vitamin C from 145±103 to 116±75 mg (p = 0.021) was observed.

High compliance of cherry intervention was observed during the 4-week intervention.

The study dose of 3 cups daily (420g) was well tolerated and achievable with 50% of the men consuming within 1 daily serving (420 g) of each other for the

duration of the 4-week intervention (see Figure 6. The 25th quartile intake was 11.2 kg; the 75th quartile intake was 11.6 kg, with a medium consumption of 11.54 kg.

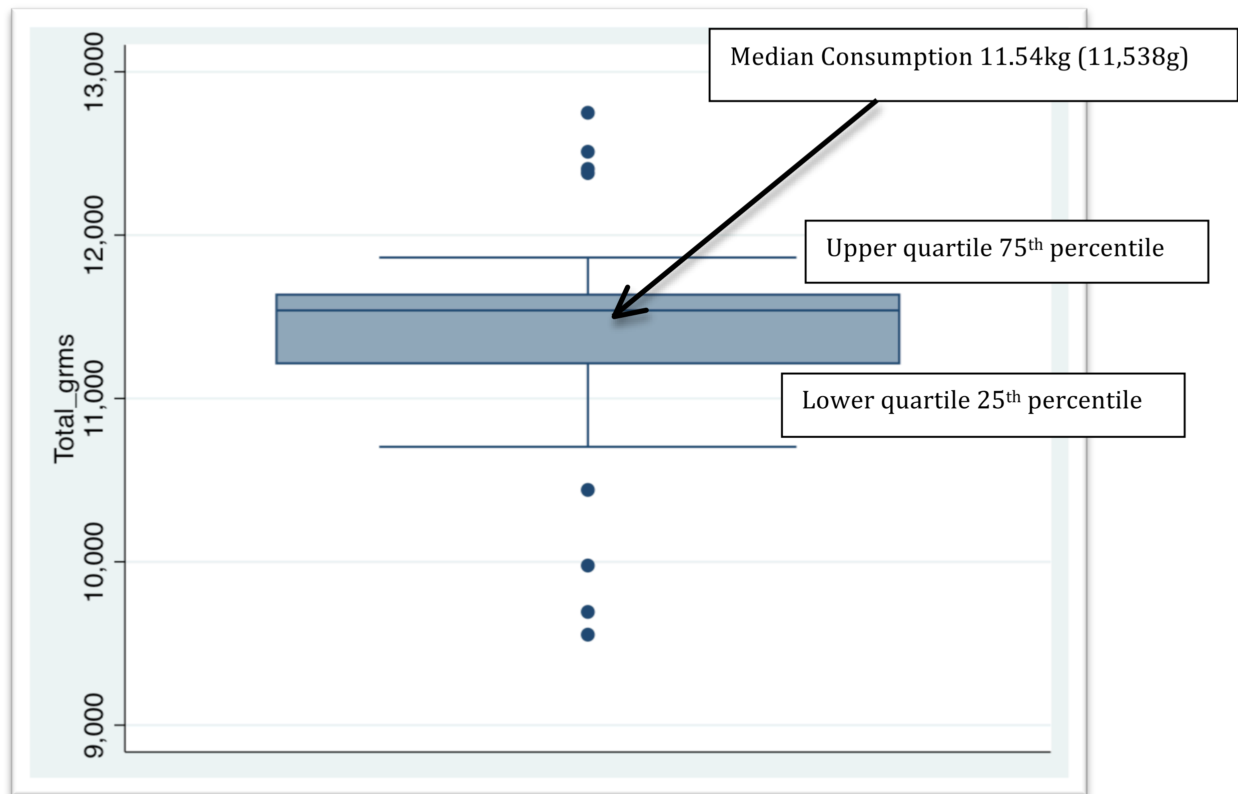


Figure 8. Participant 4-week cherry intake

Cherry consumption was not associated with changes in bowel habits or measured quality of life.

No significant changes were observed in bowel habits throughout the course of the study. Average bowel movement per day at week one and week four of the study was 1.7/day, with average bowel movements ranging from 1.7-1.8/day during the course of the study. There were no differences observed in measured quality of

life in any of the 8 SF-36 subscales (physical functioning, role-physical, bodily pain, vitality, general health, social function, role-emotions and mental health).

Daily consumption of cherries was associated with a non-significant increase in the safety biomarker, homocysteine.

The mean homocysteine value at baseline was 10.27±2.7 μmol/L and was 10.73±3.6 μmol/L at the end of the study. A slight non-significant mean increase of 0.47 μmol/L was observed ($p = 0.29$). Of importance, none of the men had values above the clinical normal range of 15 μmol/L at baseline. However, 4 of the 37 (10.8%) the men had an end of study value greater than the clinical normal range (15 μmol/L). The following participants increased from baseline to end of study: 7008 from 14.7 to 25.8 μmol/L, 7011 from 13.7 to 17.5 μmol/L, 7025 from 10.9 to 14.7 μmuol/L and 7030 from 10.1 to 16.9 μmol/L. A log transformation was not performed, given the normal distribution, see Figure 7, (median 10.2 μmol/L and mean 10.3 μmol/L). A regression model revealed no association between change in homocysteine and baseline values ($p = 0.76$). Due to the small sample size in this group, neither regression nor t-test was performed; however, slight differences in age and weight were observed in the elevated homocysteine group compared to the remaining men. Mean age was lower in the elevated group (55.1±2.9 vs. 61.8 ±7.7 years) and these men had lower baseline weights (61.8±7.7 kg vs. 103 ± 13.8 kg).

Table 3. Baseline characteristics of participants

	Participants (n = 39)
Age (Years)	61.1±7.6
Ethnicity, n (%)	
Caucasian	35 (89.7)
Other	4 (10.3)
Education, n (%)	
High School Degree	14 (35.9)
Undergraduate Degree	14 (35.9)
Graduate Degree	10 (25.6)
Other	1 (2.56)
Weight (kg)	102.5±13.4
BMI (kg/m^2)	31.5±4.3
Body Fat (%)	31.5±5.0
Waist Circumference (cm)	111.0±12.4
Hip Circumference (cm)	109.1±10.8
W:H Ratio	1.02±0.06
Systolic (mmHg)	133.6±11.0
Diastolic (mmHg)	83.4±9.2
Heart Rate (bpm)	69.4±9.9

Table 4. Comparison of inflammatory biomarkers

	Participants (n=37)			
	Before	**After**	**Change**	**p value***
PGEM2 (pg/mL)	210.61±189.51	189.71±179.43	−20.9±92.57	0.4273
TBXB2 (ng/mL)*	18.32±6.2	19.07±7.29	0.75±0.09	0.552
hsCRP (mg/L)	2.74±2.7	3.01±2.8	0.27±2.1	0.446
Homocysteine (μmol/L)	10.27±2.7	10.73±3.6	0.46±2.7	0.464

*TBXB2 average CV >10%. All *p* values are log transformed.

Table 5. Comparison of inflammatory biomarkers stratified on baseline

	Participants (n=37)			
	Before	**After**	**Change**	**p value***
PGEM2 < median (pg/mL)	78.8±29.6	90.6±44.4	11.9±45.5	0.473
PGEM > median (pg/mL)	349.7±187.6	294.3±208.7	−55.5±116.2	0.014*
hsCRP < median (mg/L)	0.9±0.5	1.5±1.1	0.6±0.9	0.011*
hsCRP > median (mg/L)	4.7±2.7	4.5±3.2	−0.1±2.9	0.302
HC* < median (μmol/L)	8.59±1.32	8.95±2.2	0.36±1.75	0.377
HC > median (μmol/L)	12.05±1.28	12.61±3.93	0.57±3.44	0.48

PGEM median (131.4pg/mL), hsCRP median (1.54 mg/L), HC median (10.2 umol/L)

Table 6. Comparison of diet composition

	Participants (n=37)		
	Baseline	**Post-Intervention**	**p value***
Energy (kcal)	2247±1339	1902±671	0.104
Fat (g)	84±49	72±29	0.128
Saturated Fat (g)	27±3	23.6±10.6	0.166
Carbohydrate (g)	277±193	240±94	0.194
Protein (g)	93±50	77±32	0.04*
Fiber (g)	26±19	22±10	0.135
Sodium (mg)	3855±2449	3349±1285	0.203
Potassium (mg)	3637±2106	3167±1335	0.083
Folate (μg)	492±295	405±175	0.048*
Vitamin C (mg)	145±103	116±75	0.021*
EPA (g)	0.7±0.1	0.5±0.1	0.101
DHA (g)	0.023±0.02	0.02±0.02	0.045*

Table 7. Circulating ACN exposure (n=30)

	Plasma					Urine				
ACN	Baseline n(%)		Final n(%)		*p*	Baseline n(%)		Final n(%)		*p*
	Undetected	Detected	Undetected	Detected		Undetected	Detected	Undetected	Detected	
C3GLU	14 (46.6)	16 (53.3)	24 (80)	6 (20)	0.006*	13 (43.3)	17(56.7)	10 (33.3)	20 (66.7)	0.25
C3RUT	27(90)	3 (10)	5 (16.7)	25 (83.3)	<0.001*	28 (93.3)	2 (6.7)	0 (100)	30 (100)	<0.001*
C3SAM	26 (86.7)	4 (13.3)	27 (90)	3 (10)	1	23 (76.7)	7 (23.3)	26 (86.7)	4 (13.3)	0.375
C3XRUT	30 (100)	0 (0)	30 (100)	3 (0)	1	30 (100)	0 (0)	30 (100)	0 (0)	1

*P value determined using McNemar's test

CHAPTER 4: SUMMARY AND CONCLUSIONS

To our knowledge, this is the first human clinical trial examining the anti-inflammatory effects of sweet cherries in a high-risk population. The current preclinical evidence suggests that bioactives in sweet cherries inhibit COX-2 mediated prostaglandin synthesis, a putative initiator to PCa.

We observed significant variability in cherry ACN content between batches and by cherry variety, consistent with the literature. The highest concentration of measured ACNs was found in the Sweet Red variety (161.56 μg/g) from Washington in late July, which contained C3RUT (122.73 μg/g), moderate amounts of C3GLU (37.68 mcg/g) and minimal amounts of C3SAM (1.15 μg/g). The second most concentrated ACN cherry variety was Bing harvested from Washington in late July, with a total concentration of 94.89 μg/g. The lowest concentration observed was in the Chelan variety from Washington at the beginning of their production season, with a total 7.18 μg/g of ACN. The greatest impact on ACN concentration was ripening time; fruit harvested in the latter part of the season had the highest levels of measured ACNs. This was illustrated by the large variation between batches from late spring/early summer to late summer. In the beginning of Washington's season, Bing cherries contained 16.88 μg/g of total ACN; whereas, during peak season at the end of July, Washington Bing's ACN content exponentially increased to 94.89 μg/g. This trend was also demonstrated in Californian Brook varieties with a 49% increase in total ACN content in 1 week. The effect of harvest time had a greater influence than that of the specific individual varieties. Minimal variation

was observed between different varieties harvested within a few weeks of each other. This trend is consistent with Serrano et al. who found an exponential increase in ACN during later stages of sweet cherry development [85].

The variability in individual cherry batch concentration of ACN resulted in significant differences in individual exposures to ACNs during the short duration of the study. Men who were enrolled into the study at peak cherry harvest were exposed to larger concentrations of ACNs (308.03 ±246.03μg) with a statistically significant increase in total ACN ($p < 0.001$), C3RUT ($p = 0.029$) and C3GLU ($p < 0.001$) concentrations during the 4-week study compared to men consented at the beginning of the cherry season (249.36±79.14μg). This trend is also observed in typical American diets, as it has been estimated that ACN intake is higher in the summer, 215 mg/day, than the winter, 180mg/day [86]. This effect could be in part due to the amplification of ACN, such as in cherries and other fruits and vegetables in peak harvest time [25].

Concentration of total ACN in cherries in this study was lower than previously reported; however, this is the first time the preferred detection method of LC-MS/MS has been used to analyze ACN concentration in sweet cherries to our knowledge. The USDA Database for the Flavonoid Content of Selected Foods estimates sweet cherries ACN content at 80mg/142g [47]. Other studies have reported sweet cherries ACN content between 60-147mg/100g of ACN, depending on seasonality and variety [25]. However, methodology to determine total phenolic concentration varies dramatically. Chaovanalikit and Wrolstad, using the pH-

differential method via UV spectrophotometer, found sweet cherries to contain 2.2 to 60.6mg/100g, dependent on the flesh color [49]. Whereas, Serrano et al. using HPLC, found a larger variation between sweet cherry cultivars, ranging from 10 – 200mg/100g of ACN, Brooks having the lowest concentration [50]. Valero et al. (also using HPLC) detected a range of 18.56 – 81.89 mg/100g of ACN, differing between cultivars [87]. Notably, HPLC has limited sensitivity to determine metabolites and conjugates, particularly at extremely low concentrations [75], as seen in sweet cherries.

In our study the circulating levels of ACN metabolites were low, as determined by the limits of detection for LC-MS/MS methodology to measure the 4 major ACNs including C3RUT, the highest concentrated ACN found in cherries. The study protocol to measure the metabolites of ACNs in urine and plasma in the current study had limitations. ACN metabolite levels peak in circulation 1-4 hours post ingestion and are rapidly excreted within 6 hours of consumption and are optimally captured in repeated sampling over short interval based pharmacokinetic studies [88]. To account for this, participants were instructed to eat their final serving of cherries 1-2 hours before the final blood draw and participants that consumed the final serving greater than 4 hours were eliminated from the analysis. Yet this limited 3-hour timespan still resulted in variability in plasma ACN concentrations. Overall circulating plasma levels of the primary ACN in cherries, C3RUT, were low. However, the concentration of C3RUT increased significantly at the end of the study as compared to the beginning. Urinary excretion was similar,

with significant increases in C3RUT excretion at the final visit. Of note, we observed a significant decrease in plasma C3GLU post intervention. This may be due, in part, to the timing of the blood draw. To minimize blood draws in our study, plasma was drawn pre-washout, possibly capturing residual C3GLU, the most common Cy in American's diet, prior to the washout period. Further complicating detection are the multiple ACN metabolites observed throughout metabolism, such as gluconoridated and methylated derivative's [52]. It is estimated that the major metabolites (68-80%) of ACN are metabolized via glucuronidation, methylation and sulfation. LC-MS/MS detection would not pick up these metabolites in the plasma and urine, possibly underestimating the exposure of other ACNs in the current study. However, after controlling for last cherry consumption time, our results suggest high inter-individual variation in ACN metabolite levels that may reflect the influence of individual variation in metabolism of ACN bioactives.

The current research revealed a significant reduction in PGEM in men with elevated baseline values; however, no change was observed in men with mean baseline values below the median. In men with baseline values less than the median (131.4 pg/mL), a significant reduction of -55.5 pg/mL was observed. This lends support to an inhibitory effect of cherry consumption on COX-2 mediated prostaglandin synthesis in participants who initiated the study with elevated prostaglandin levels, a group of men more likely to benefit from inhibition of this pathway. This trend is often noted in bioactive human trials, as markers of stress or damage are low in healthy volunteers, and resulting effects are difficult to measure

[89]. Broncel et al. examined the effects of 300mg ACN (Aronia extract) daily for 2 months on inflammation markers (including hsCRP but not PGEM) in healthy volunteers versus those with existing metabolic syndrome. Results showed a significant reduction in only one inflammatory marker, Thiobarbituric Acid Substances (TBARS), a measure of oxidative stress and two CVD markers (LDL and blood pressure) in the metabolic syndrome group, and no significant changes in the healthy cohort [90].

Similar to PGEM, hsCRP levels revealed a significant change when stratified on the median of the baseline value. A significant increase in hsCRP was noted in men with baseline values below the median; however, this rise did not extend the mean hsCRP of this cohort above the level associated with increased CVD risk [91]. Comparably to PGEM, ACN intake exposure or circulating ACN exposure was not associated with hsCRP outcomes. Several studies have assessed the effects of ACN on hsCRP, revealing unimpressive results. Kaspar et al. examined daily consumption of 150 g of purple vs. white potatoes for 6 weeks in 24 healthy men and found a non-significant decrease in hsCRP [92]. In a parallel-double blinded study, Curtis at al. examined the effects of daily intake of 500mg of ACN (elderberry) on CVD markers, including hsCRP in 52 healthy premenopausal women and did not find any significant reductions after the 12-week intervention [93]. Kelley et al., found a significant reduction in hsCRP in 18 healthy adults after consumption of 280g of sweet cherries for 28 days, however the study design had limitations. The author cited a decrease in circulating CRP in 12 of the 18 subjects, and this effect

was only statically significant at day 28, with a modest reduction of 0.5 mg/L, and regression to the mean by day 35. The study examined several other markers of inflammation (ICAM, NO, IL-6 and RANTES) and CVD risk factors (LDL, TG, HDL and VLDL), and the only significant effect was a slight reduction of RANTES at day 28 [41].

Although chronic use of ACN on circulating hsCRP concentrations has produced inconsistent results, postprandial effects following ACN intake on hsCRP has proven more beneficial. In a crossover study, Eldirsinge et al. examined the impact of a strawberry ACN beverage verses a placebo following a high carbohydrate, high fat meal in 24 overweight adults. They found a significant reduction (-0.4 mg/L , $p = 0.02$) in hsCRP 6 hours following consumption [94]. The sweet cherries in the current study may have decreased the influx of postprandial inflammation directly following insult of food consumption, but did not attenuate levels on a chronic basis.

The current research found no overall significant change in inflammatory markers, PGEM, TBX2 and hsCRP from baseline to end of study. This is consistent with the current cherry literature. Several studies have examined cherry, specifically tart cherry juice effects on post exercise inflammation in healthy populations, finding positive out comes. Unfortunately, many of the studies that claim inflammatory protection have multiple limitations, leading to inconclusive results. Conolly et al. examined the effects of 12oz of tart cherry juice twice daily for 8 days on 14 male athletes, finding a reduction in perceived pain and attenuated

measured elbow strength loss [42]. This study did not assess circulating biomarkers (e.g. hsCPR or IL-6) and the two outcome measures resulting in significant improvements contain participant and investigator measurement biases because the pain was self-report and the investigators were not blinded. Additionally, the perceived pain scale was only significant at hours 24 and 48 following the exercise (not at 72 or 96 hours following) and merely differed by 1 to 2 points on a 0-10 point scale, showing a minimal relevant outcome [42].

Two studies investigated the effects of tart cherry juice post long distance running. In an extremely limited study design, Kuehl et al. assessed the difference between tart cherry juice and a placebo's effect on perceived pain post race as well as participant's satisfaction with the beverages. The researchers observed a significant decrease in pain as well as increased satisfaction with consumption of the tart cherry juice. [95]. Howatson et al. also examined tart cherry juice consumption for 5 days before the prior and 48 hours post race in 20 recreational runners. They assessed circulating plasma markers of muscle damage (IL-6, CRP, TBARS and protein carbonyls) and measured recovery isometric strength. The study concluded an improvement in isometric strength following the race. Yet no significant changes in any of the circulating biomarkers were detected [96]. Similarly, Bowtell et al. explored the effects of tart cherry juice on muscle damage following 10 sets of single-leg knee extensions in 10 male athletes. The primary outcomes were knee extension maximum voluntary contractions (MVC) and circulating biomarkers (creatine kinase, nitrotyrosine, hsCRP, total antioxidant

capacity and protein carbonyls). The results revealed only significant changes in MVC recovery time and a slight lessening in the increase of protein carbonlys observed after the run [97]. A majority of the current evidence in human trials is limited to tart cherry juice and is typically restricted to changes in perceived inflammation rather than the effect on circulating biomarkers of inflammation.

Four or 10.8% of the participant's final serum concentration of homocysteine increased outside of the clinical normal range of 15 μmol/L, whereas at baseline, before the cherry intervention, values were all within the normal clinical range. Although this increase was not significant it maybe of concern, as epidemiologic evidence suggests mild hyperhomocysteine as a modest predictor of CVD and stroke [98]. The members of the Homocysteine Studies Collaboration concluded that a 25% reduction in homocysteine concentration is associated with an 11% lower risk of ischemic heart disease (0.89 odds ratio, 95% CI: 0.83-0.96) and 19% lower risk of stroke (0.81 odds ratio, 95% CI: 0.69 -0.95) [76]. In contrast, recent clinical prospective trails have lead to minimal impact on cardiovascular disease [98]. The Norwegian Vitamin (NORVIT) trial and the Heart Outcomes Prevention Evaluation (HOPE) 2 trial both examined secondary prevention treatment with B vitamins to lower homocysteine levels, resulting in lower cardiac events [78, 79]. The NORVIT trial included 3,749 adults post acute myocardial infarction (MI), randomly assigned to 1 of 4 treatments daily: 0.8 mg folic acid, 0.4 mg vitamin B12 and 40 mg of vitamin B6; 0.8 mg folic acid and 0.4 mg vitamin B12; 40 mg vitamin B6 or placebo for 40 months. The primary outcome was MI reoccurrence. Mean homocysteine

level was lowered by 27% in the folic acid plus B vitamin group; however a lower risk of recurrent CVC was not found [78]. The HOPE-2 trial enrolled 5,522 participants with a history of CVD or diabetes randomly to either 2.5mg folic acid, 50mg vitamin B6 and 1mg of vitamin b12 or placebo for 5 years; primary outcome was death fro CVD causes, MI and stroke. Similarly, they found a significant reduction in plasma homocysteine but not a reduction in the risk of major cardiovascular events [79]. The Vitamin Intervention for Stroke Prevention (VISP) Trial had a similar structure; however its primary outcome was reduced risk of stroke. VISP randomly enrolled 3,680 participants status post cerebral infarction to either a high dose of 2.5mg folic acid, 25 mg vitamin B6 and 0.4mg vitamin B12 or low does of 20 μg of folic acid, 200 μg vitamin B6 and 6 μg vitamin B12. Mean homocysteine was reduced by 2 umol/L in the high dose group; however, no treatment effect on the end point was observed in either group was [77]. Although the clinical data remains inconsistent, the elevation of homocysteine observed in the current study is of concern, given the study's high-risk population.

The high-risk study population, defined as older, overweight men was recruited successfully. However, the sample was highly educated and ethnicity was limited to Hispanic and non-Hispanic white, not necessarily representing the general population at risk for PCa. Incidence rates of PCa in black U.S. men compared to white men are nearly double (233.8 per 100,000 compared to 149.5 per 100,000 respectively). Mortality rates follow this trend, with black males dying from PCa twice as often as white men. Conversely, U.S. Hispanic men have a slightly

lower incidence and mortality rates than white men [99]. Lower socioeconomic status, tied tightly to education, is also considered a risk factor for enhanced PCa mortality [100]. Although the study population was highly educated, 35.9% earning a college degree and 35.9% attaining a graduate degree; further decreasing the homogeneity of the study population compared to the general at-risk population.

During the intervention the study population had high compliance to the study dose, minimal alterations in intake, and consistent bowel movements during the intervention period. The study dose of 420 g per day was well tolerated and 50% of the men in the study consumed within 1 daily serving of each other. Bowel movements remain consistent at 1.7 per day during the 4-week intervention. Minimal diet alterations were noted including a significant decrease in protein, as well as a significant decrease in folate and vitamin C. Folate decreased by -86.9 ± 258.6 µg/day and vitamin C by -29±73 mg/day ($p = 0.021$). However, the decrease in folate and vitamin C are not physiologically relevant to see biological effects, as the DRI for vitamin C is 75mg/day and 320 µg/day for folate [101] and the participants intake of folate and vitamin C surpassed the DRI during the intervention period (116±75 mg and 405±175 µg respectively). Also, previous studies recommend the use of 1,000mg vitamin C or 2.5 mg/day of folate to observed clinical outcomes (e.g. lowering of homocysteine) [77, 102].

The results of this study should be interpreted with caution and limitations should be noted. Since a 'normal' level of inflammation has yet to be established,

the median PGEM was utilized as the cut point for elevated values. Further research is needed to determine the average normal values. The sample size was modest, as this was a pilot study. Our results must be replicated at a larger scale, preferably in at-risk population, such as men with elevated inflammatory markers. NSAID use may have affected the outcomes, as 13.5% of the men were chronic NSAID users prior to the study and discontinued use at washout. This may have caused COX-1 and COX-2 metabolites to rebound during the course of the study, complicating the biomarker results. Finally, this study examined a single food approach. Clinical trials of single agents have yielded disappointing results [103]. Since carcinogenesis is a multistage process in which many normal cellular pathways become irregular [10], it is unlikely that one agent could prove effective in preventing cancer. A combination of naturally occurring chemopreventive substances on a chronic basis is recommended to prevent or slow down the development and progression of PCa.

Based on our findings, we conclude that 1c (142g) of sweet cherries 3 times daily for 4 weeks significantly reduced the COX-2 metabolite, PGEM, in men with elevated baseline levels. This was the first study to examine the chronic effects of daily sweet cherries on COX-2 inhibition in a population of men at elevated risk for PCa. The effects of cherries are likely attributed to its high concentration of ACN, specifically Cy. Given the modest anti-inflammatory effect noted in the current study with moderate amounts of the whole food, it would be advantageous to determine the optimal dose of Cy necessary to yield anti-inflammatory properties in the chemoprevention setting that have been observed in cell and animal models.

However, with the strong effect of season on ACN concentrations, attempts to produce a more equivalent exposure per subject such as dried or frozen product from the same batch is recommended in further studies. Further, while not significant, we find it concerning that homocysteine levels were elevated above clinical norms in 10% of subjects with lower body weight consuming 3 cups of cherries for the short duration of 4 weeks, a pattern observed in animal studies. Additional efforts are needed not only to assess the beneficial effects of cherries and other ACN containing foods but also to assess the potential adverse effects of higher doses of ACN intakes within ongoing trials on homocysteine levels.

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