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A pilot sweet cherry feeding study in overweight men: Tolerance, safety, and anthocyanin exposure

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ABSTRACT

Sweet cherries are rich in bioactive anthocyanins (ACN) and thus are considered a functional food. Yet, tolerance to and bioavailability of a significant daily dose of cherries has not been evaluated in humans. This pilot study aimed to assess compliance, tolerance, safety, and change in circulating/excreted ACNs due to daily consumption of sweet cherries. Change in bowel habits, quality of life, urinary/circulating concentration ACNs, and inflammatory biomarkers were measured in overweight, older men (n = 37) before and after daily consumption of three cups of sweet cherries for 4 weeks. Cherry fruit ACN content was also measured. Tolerance to intervention was high in this study. An increase in several plasma/urine ACNs was observed, but did not correlate with the amount of ACN measured in the fruits. No significant changes in inflammatory biomarkers were observed. This intervention was feasible and increased anthocyanin exposure in overweight men.

1. Introduction

Sweet cherries (*Prunus avium L.*) are commonly consumed in the U.S. and contain several bioactive compounds, including polyphenols (McCune, Kubota, Stendell-Hollis, & Thomson, 2011). As such, sweet cherries are considered a functional food with the potential to improve human health by modifying inflammation and oxidative stress (Kelley et al., 2013; Kelley, Rasooly, Jacob, Kader, & Mackey, 2006).

Anthocyanins (ACNs) are the major class of polyphenols in cherries (Kelley et al., 2013; Tapiero, Tew, Ba, & Mathe, 2002). Cyanidin is the major ACN found in cherries (Ferretti, Bacchetti, Belleggia, & Neri, 2010). The primary cyanidin found in sweet

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cherries is cyanidin-3-rutinoside (C3RUT), followed by cyanidin-3-glucoside (C3GLU), with low levels of other ACNs also present (Kim, Heo, Kim, Yang, & Lee, 2005; Saric et al., 2009). Additionally, protocatechuic acid (PCA) is a common metabolite of cyanidin in humans (Vitaglione et al., 2007).

Sweet cherries and their ACNs are being investigated for use in cancer, cardiovascular disease, and other inflammation-related diseases. In animal models, cherry bioactives reduce biomarkers of inflammation, including those mediated by cyclooxygenase (COX)-2 (He et al., 2006; Hou, Yanagita, Uto, Masuzaki, & Fujii, 2005; Piccolella et al., 2008; Tall et al., 2004). A few small, human studies suggest that cherry consumption may modify inflammatory biomarkers, such as C-reactive protein (CRP), in healthy adults (Connolly, McHugh, Padilla-Zakour, Carlson, & Sayers, 2006; Kelley et al., 2006, 2013). On the other hand, rats administered a single oral dose of 100 mg of ACN have been reported to experience a near-immediate rise in homocysteine levels (Nakagawa, Maruyama, & Miyazawa, 2002), indicating that over-exposure to ACNs is potentially problematic. Taken together, studies are limited and data are insufficient to evaluate the safety and efficacy of regular, higher-dose intake of sweet cherries and other ACN rich functional foods in humans.

Our primary aim was to measure compliance, tolerance, and change in plasma/urine ACN levels in response to daily intake of three cups of fresh, sweet cherries for 4 weeks. Our secondary aim was hypothesis generating and was to determine if there was any evidence of a change in biomarkers of inflammation in response to ACN dose. When designing human feeding studies with whole foods it is imperative to evaluate a reasonable dose for regular intake as well as a dose that is likely to have biological benefit. This is especially true as ACNs may have a dose-dependent effect on metabolism (Carkeet, Clevidence, & Novotny, 2008). Since humans consume whole foods and there is some argument that the synergy of bioactives in whole foods may outweigh benefits of any single bioactive compound, we sought to test whole cherry feeding. It is well-recognized that the concentrations of ACN will vary in relation to storage conditions and ripening of the cherry, as well as cultivar selected (Goncalves et al., 2004) and so we also measured ACN content in the cherries provided to our study participants in order to control for variable ACN levels. To our knowledge, this study provides the first preliminary data on a commonly consumed function food's tolerance, safety and ability to increase ACN exposure in humans that will inform future studies on this fruit.

2. Subjects and methods

2.1. *Participant eligibility and study design*

This feeding trial was conducted in relatively healthy older, overweight/obese men because this population exhibits mild basal inflammation (Kantor, Lampe, Kratz, & White, 2013) as compared to the normal-weight population. Respondents (n = 105) to print, television, and electronic advertising were screened via telephone for eligibility (Fig. 1). Inclusion criteria were men age >50 years, body mass index (BMI) 25–45 kg/m^2, abstinence from tobacco products, willingness to discontinue dietary supplements with the exception of a multivitamin for 1 week prior to enrollment and during the trial, willingness to discontinue anti-inflammatory medication with the exception of low-dose aspirin, absence of co-morbidities, and cancer-free >12 months. The most common exclusion criterion was a history of major chronic illness (n = 12). Written informed consent was obtained from all study participants prior to enrollment (n = 39). Of these, 37 successfully completed the washout period. The University of Arizona Institutional Review Board approved this study protocol. The study was conducted between May and August of 2011.

Upon consent, participants began a 1-week washout where they were instructed to avoid foods rich in ACNs and limit consumption of fruits and vegetables to ≤5 servings/day (Fig. 1). Next, participants began the 4-week intervention of consuming a one-cup (142 g) serving of sweet cherries three times per day, while still limiting intake of other fruits and vegetables to ≤5 servings/day. Subjects were advised to maintain regular body weight, diet, and physical activity levels throughout the study.

2.2. *Compliance, tolerance, and safety*

Participants returned the uneaten cherries, pits, and stems to the clinic weekly, where waste weight was subtracted from the weight of cherries provided to each participant weekly to estimate compliance to the intervention. To measure tolerance, participants completed a quality-of-life questionnaire (MOS 36-item short-form survey; SF-36) (Ware & Kosinski, 2001) at baseline and end-of-study, and bowel habits were recorded throughout the intervention using a previously validated daily bowel habits questionnaire (BHQ) (Bassotti et al., 2004). To measure safety, serum levels of homocysteine were measured at baseline and end-of-study. Samples were processed at Lab Corp (Tucson, AZ, USA), which defined an abnormal value as >15 μmol/L.

2.3. *Anthropometrics*

Anthropometry was performed at baseline and end-of-study by a registered dietitian. Waist and hip circumference were measured using previously published methods (Lohman, Roche, & Martorell, 1988). Body fat was estimated using a handheld Omron Body Fat Analyzer HBF-306 (Omron Healthcare, Inc., Vernon Hills, IL, USA). Resting blood pressure was measured in duplicate by an automatic blood pressure monitor by ReliOn HEM-780REL (Omron, Inc., Bannockburn, IL). The average of two systolic and diastolic blood pressure (mmHg) and heart rate (beats/min) measurements were recorded.

2.4. *Dietary intake analysis*

Dietary intake was assessed using the previously validated Arizona food frequency questionnaire (AFFQ) (Thomson et al., 2003) at baseline (estimated dietary intake in the past 3 months) and end-of-study (estimated dietary intake during the 4-week intervention). Metabolize (the proprietary dietary data reduction system developed at the University of Arizona, Tucson, AZ, USA) was used to determine average daily food/nutrient intake.

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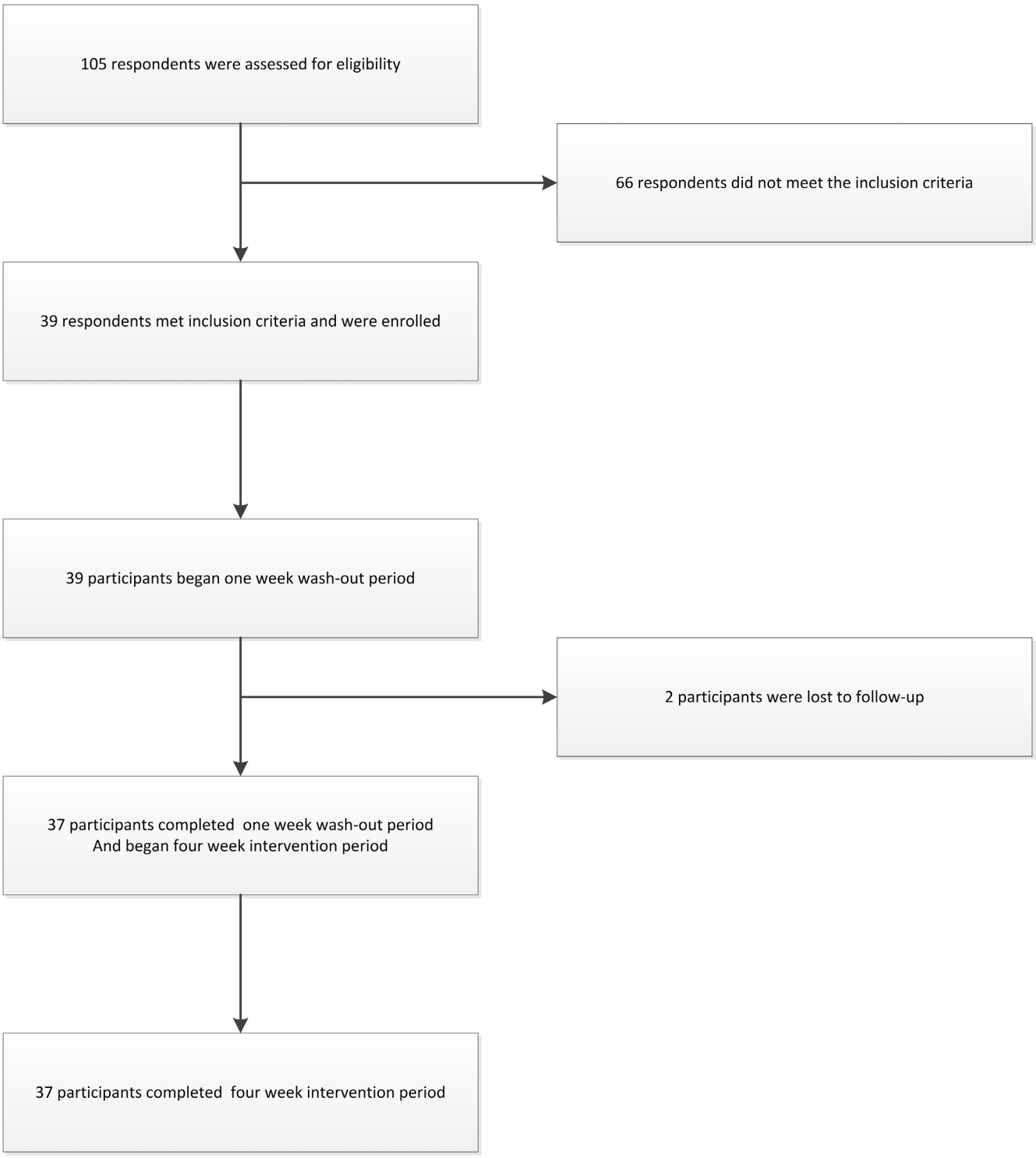

Fig. 1 – Consort diagram of the sweet cherry feeding study. Of the 105 respondents, 66 did not meet the eligibility requirements. The resulting 39 that met the inclusion criteria were enrolled and began the 1 week washout period where they avoided anthocyanin rich foods and limited fruit/vegetable intake. Two participants were lost during washout and so, after washout, 37 participants began the 4 week intervention period. The intervention was consumption of three cups of sweet cherries per day while continuing to avoid other high anthocyanin foods. All 37 participants completed the 4 week intervention period. Measurements were taken at baseline, during the 4 week intervention period and at the end-of-study.

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2.5. Specimen collection and processing

Non-fasting serum and plasma samples were collected from the subject's antecubital vein 1–2 h following cherry consumption using standardized protocols (stored at –80 °C). Twenty-four hour urine was collected using standardized protocols and was stored at approximately 4 °C throughout the collection period using a personal cooler with ice that was stored in each participant's refrigerator. Plasma and urine samples utilized to quantify ACN concentration were diluted with formic acid (FA) to a final concentration of 5% prior to freezing.

2.6. Sweet cherry processing

The Oregon Sweet Cherry Commission and the Washington State Fruit Commission supplied the sweet cherries. Cherries were delivered weekly, weighed, and separated into daily portions of 3 × 142 g/serving (426 g total/day), assembled into 7-day packages for each subject, and stored at 9 °C. Subjects picked up cherries weekly at the clinical research facility at The University of Arizona (Tucson, AZ, USA), were instructed on storage conditions and then transported them home in a cooler and placed into their home refrigerator.

2.7. Phenolic concentrations in urine and plasma

Seven men were excluded from plasma and urine analyses, because their final serving of cherries was consumed >4 h prior to their final visit, yielding a final sample size of 30 for these analyses. Quantification of ACNs/PCA in plasma and urine was conducted via liquid chromatography mass spectrophotometry/mass spectrophotometry (LC–MS/MS; Biomedical Mass Spectrometry Lab, Ohio State University, Columbus, OH, USA) (Ling et al., 2009; Mallery et al., 2011). Urine standards were acidified with 5% FA to be consistent with the samples before undergoing LC–MS/MS. The LC–MS system consisted of a Finnigan TSQ Quantum EMR Triple Quadrupole mass spectrometer (Thermo Fisher Scientific Corporation, San Jose, CA, USA) and a Shimadzu HPLC system (Shimadzu, Columbia, MD, USA), equipped with a CBM-20A system controller, LC-20 AD pump, SIL-20AC autosampler, CTO-20A column oven, DGU-20A5 degasser, and FCV-11AL valve unit. The temperature of the auto-sampler was set at 4 °C during operation. All operations were controlled by Finnigan Xcalibur software (Home Page Version 1.4 SR1).

For ACN analysis in plasma, both standards and samples were protein crushed by 1 mL acetonitrile, precipitated, and the supernatant was separated by centrifugation and dried under N_2 stream. Samples were reconstituted in 100 μL 25% acetonitrile and 0.1% FA and filtered (0.45 μm microfilter) prior to LC–MS/MS analysis. For ACN analysis in urine, standards and samples were processed by solid phase extraction. Oasis HLB cartridges were sequentially pre-conditioned and washed after urine samples were loaded. In the final step, ACNs were eluted by MeOH and 0.1% FA. The eluted fraction was dried under N_2 stream, and then the residue was reconstituted in 100 μL 25% acetonitrile and 0.1% FA. For PCA analysis in plasma and urine, both standards and samples were extracted with ethyl acetate (10:1 v/v), and then the organic layer was separated by centrifugation and dried under N_2 stream. The samples were reconstituted in 100 μL 50% acetonitrile and 0.1% FA and filtered (0.45 μm microfilter) prior to LC–MS/MS analysis. Calibration curves were linear from 1 to 1000 ng/mL and from 1 to 500 ng/mL with a regression coefficient of >0.99 for urinary and plasma ACNs and PCA, respectively. Quality controls prepared in triplicate at concentrations of 5 and 50 ng/mL were inserted throughout both blood and urine runs to ensure data quality. Level of dictation was set at 1 ng/mL for all sample types.

2.8. Phenolic concentrations in sweet cherries

Pitted cherries with stems removed were obtained from each weekly batch and divided into 100 g samples, snap frozen with nitrogen vapor, and stored at –80 °C until analysis. A total of 11 batches of cherries were used in the intervention and all 11 were analyzed for C3GLU, C3RUT, cyanidin 3-sambubioside (C3SAM), and cyanidin 3-(2(G)-xylosyl) rutinoside (C3XRUT) content throughout the growing season via LC–MS/MS, as described earlier (Ling et al., 2009; Mallery et al., 2011).

2.9. Inflammatory biomarker measurements

Urinary PGEM was detected using the PGEM ELISA kit from Cayman Chemical (Ann Arbor, MI, USA; catalog #514531; lower limit of detection: 2.0 pg/mL). The standard developed from this kit had a final concentration of 1000 pg/ml (from a 40 ng/ml bulk standard diluted 4 parts into 76 parts kit provided-buffer and 24 parts carbonate buffer). Samples were prepared by diluting 500 μL of urine in 150 μL of carbonate buffer, 200 μL of phosphate buffer and 150 μL of kit provided-buffer. Standards/controls/samples were added in duplicate to a 96-well plate coated with mouse anti-rabbit IgG. PGEM anti-serum and acetylcholinesterase tracer were added to the appropriate wells and incubated for 18 h at room temperature. After washing, Ellmans reagent was added and the absorbance was read at 412 nm.

Urinary Thromboxane B2 (TBX2) was detected using the TBX2 Metabolite ELISA kit from R&D Systems (catalog #KGE011; lower limit of detection: 0.214 ng/mL). Standards, controls, or 10-fold diluted samples were plated in duplicate on a 96-well microplate coated with goat-anti-mouse polyclonal antibody. Primary mouse monoclonal antibody was then added and incubated for 2 h at room temperature. Horseradish peroxidase-conjugated TBX2 was then added and incubated for another hour at room temperature. After washing, substrate solution was added, the plate was incubated for 30 min at room temperature and then the absorbance was read at 450 nm.

Serum high-sensitivity CRP was measured using the High Sensitivity CRP ELISA kit from US Biological (Salem, MA, USA; catalog #C790-20H; lower limit of detection: 0.1 mg/mL). Ten microliters of patient serum or standard was added in duplicate to a 96 well plate that was pre-coated with mouse monoclonal anti-CRP. One hundred microliters of horseradish peroxidase-conjugated anti-CRP was added to each well and incubated for 45 min at room temperature. After washing, 100 μL of the kit provided substrate was added and then the plate was incubated for 20 min at room temperature. The stop solution was added and then the absorbance was read at 450 nm.

For all of the earlier assays, paired samples were run on a single plate. Standards and controls were prepared following

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the manufacturer's instructions. Data were considered valid if the intra-assay coefficient of variation was <10%.

2.10. Statistical analysis

Average daily kilocalorie (kcal) and percent energy intake from the sweet cherry intervention were calculated by averaging the measured amount cherries consumed per day in the last week, multiplying ~0.78 kcal/g of cherry weight (from the AFFQ estimates), and then dividing the total number of cherry-derived kcal by end-of-study AFFQ-calculated total kcal. Participant characteristics, dietary characteristics, bowel habits (day 1 versus the average of the last 5 days' defecation frequency while on study; nine participant did not have complete BHQ records), SF-36 scores, ACN urine/plasma levels, and all inflammatory biomarker values at baseline were compared to end-of-study values using Wilcoxon signed-rank tests due to the skewed distributions. Baseline and end-of-study plasma/urine ACN levels were compared using McNemar's test on the presence or absence (below the limit of detection) of ACNs in plasma/urine. The measured total ACN exposure from cherries in the last week of intervention was divided by 7 to estimate the amount of ACNs each participant was exposed to in the final 24-h preceding the end-of-study sample collection and will be called "end-of-study cherry ACN exposure". This end-of-study cherry ACN exposure was compared to end-of-study plasma/urine ACN and inflammatory biomarker values using Spearman correlation. Sensitivity analyses were conducted by performing all analyses with and without participants who reported >1000 kcal/day change in either direction from baseline to end-of-study ($n = 6$). All statistical analyses were performed using Stata 12.0 (StataCorp, College Station, TX, USA).

3. Results

3.1. Baseline and change in study population characteristics

The pilot study population comprised well-educated men between 50 and 83 years of age who were obese or overweight (Table 1). Of the participants, 4 (10.8%) were Hispanic; the remaining 33 (89.2%) were non-Hispanic white. Median waist circumference increased at end-of-study ($P = 0.05$; from 111.8 to 113.0 cm on average), but there were no significant changes in other, related measurements (i.e. BMI and weight) (Table 2).

3.2. Compliance, tolerance, and safety

The study dose of three cups (426 g) of sweet cherries per day was well tolerated and achievable, with 75% of the men consuming >400 g on average for the duration of the 4-week intervention. Specifically, median daily cherry intake in grams, kcal and % total kcal was 415 g/day, 321 kcal/day and 17.2% of the total kcal/day, respectively. Median total anthocyanin exposure from sweet cherries was measured at 6.77 mg/day during the last week of intervention.

We observed no significant changes in self-reported bowel habits throughout the intervention (median number of defecations = 1.4 on day 1 and at end-of-study, $P = 0.452$), or in measured quality of life in any of the SF-36 subscales throughout the intervention (median general SF-36 score = 82 for both baseline and end-of-study, $P = 0.264$). Further, no other adverse gastrointestinal effects were reported. No significant change in serum homocysteine levels was observed overall ($P = 0.531$, Table 3). There was some evidence of a change in diet composition between baseline and end-of-study. Dietary intake of fat, protein, and total energy (kcal) decreased significantly ($P = 0.03$, $P = 0.01$ and $P = 0.04$, respectively; Table 2). Although participants were advised against changing their dietary habits (except for avoiding high anthocyanin foods and limiting fruit/vegetable intake throughout washout and the duration of the study), participants reported a decrease in intake of meal replacement drinks, milk and milk-alternative drinks from baseline to end-of-study. However, weight and BMI remained stable from baseline to end-of-study.

Table 1 – Participant characteristics at baseline ($n = 37$), mean ± standard deviations (SD) and median (25th, 75th percentiles), or number (n) percent of total (%).

Characteristic	Mean ± SD	Median (25th, 75th)
Age (y)	61.4 ± 7.7	60.0 (56.3, 64.2)
Weight (kg)	103.0 ± 12.9	104.1 (91.8, 110.0)
BMI (kg/m^2)	31.7 ± 4.3	31.3 (28.1, 34.2)
Body fat (%)	31.8 ± 5.0	31.8 (28.2, 34.6)
Waist circumference (cm)	112.0 ± 11.8	111.8 (104.1,118.1)
Hip circumference (cm)	109.5 ± 10.5	106.7 (101.6,116.8)
Waist:hip ratio	1.02 ± 0.05	1.02 (1.00, 1.05)
Systolic blood pressure (mmHg)	134.5 ± 10.6	134.0 (128.0,141.0)
Diastolic blood pressure (mmHg)	83.2 ± 9.4	85.0 (78.0, 90.0)
Heart rate (bpm)	69.1 ± 9.8	68.0 (63.0, 75.0)
		n (%)
Ethnicity		
Non-Hispanic white		33 (89.2)
Other		4 (10.8)
Highest education reported		
High school degree or below		2 (5.4)
Some undergraduate school or undergraduate degree		19 (51.4)
Some graduate school or above		16 (43.2)

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

Individual ACN content by cherry variety and batch was highly variable (Fig. 2). Large batch-to-batch variability was observed for total ACN, C3GLU, and C3RUT. C3XRUT was not detected in any of the batches. The concentrations of C3GLU, C3RUT, and C3SAM generally increased toward the end of the harvest season for each varietal.

3.4. Significant increases in circulating and excreted anthocyanin were noted post-cherry consumption

Both plasma and urinary C3RUT increased with sweet cherry consumption (both $P \leq 0.01$; median changes 2.04 and 64.17 ng/

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Table 2 – Comparison of participants and dietary characteristics (from food frequency questionnaire) at baseline and end-of-study ($n = 37$), median (25th, 75th percentiles).

Participant or dietary characteristic	Baseline	End-of-study	P-value[a]
Weight (kg)	104.1 (91.8, 110.0)	104.1 (92.3, 110.0)	0.076
BMI (kg/m^2)	31.3 (28.1, 34.2)	31.3 (28.1, 34.5)	0.480
Body fat (%)	31.8 (28.2, 34.6)	30.8 (25.2, 35.1)	0.763
Waist circumference (cm)	111.8 (104.1, 118.1)	113.0 (104.1, 119.4)	0.048
Hip circumference (cm)	106.7 (101.6, 116.8)	107.9 (104.1, 116.8)	0.162
Waist:hip ratio	1.02 (1.00, 1.05)	1.02 (0.98, 1.05)	0.873
Systolic blood pressure (mmHg)	134.0 (128.0, 141.0)	132.0 (127.0, 137.0)	0.137
Diastolic blood pressure (mmHg)	85.0 (78.0, 90.0)	80.0 (75.0, 88.0)	0.385
Heart rate (bpm)	68.0 (63.0, 75.0)	69.0 (61.0, 79.0)	0.172
Energy (kcal/day)[b]	2012.1 (1585.4, 2483.5)	1850.0 (1360.1, 2244.5)	0.038
Protein (g/day)[b]	80.3 (67.4, 101.2)	70.2 (54.8, 93.6)	0.013
Carbohydrates (g/day)[b]	253.7 (198.4, 303.3)	233.0 (179.4, 306.7)	0.316
Total fat (g/day)[b]	71.3 (55.1, 94.7)	67.9 (48.9, 85.2)	0.029
Saturated fat (g/day)[b]	23.7 (18.6, 31.9)	22.3 (16.6, 29.3)	0.084

[a] P-value as determined by Wilcoxon signed-rank test.
[b] Includes estimated dietary contributions from the intervention sweet cherries as reported by participants on the food frequency questionnaire (end-of-study only).

mL, respectively; Table 4). Plasma C3GLU decreased very slightly ($P = 0.01$) while urinary C3GLU increased to a greater, but non-significant, magnitude ($P = 0.25$). Consistent with low abundance in sweet cherries, very few participants had measurable C3SAM or C3XRUT in urine or plasma. There was no evidence of change in PCA concentrations nor was there was evidence of a correlation between end-of-study cherry ACN exposure and end-of-study total circulating ACNs or PCA in the plasma or urine (data not shown).

3.5. *End-of-study changes in inflammatory biomarker levels were dependent on baseline biomarker and body mass index values*

We observed no overall effect of the intervention on inflammatory biomarkers (Table 3). Only participants with lower baseline urinary TBX2 levels demonstrated a slight increase in this biomarker at end-of-study ($P = 0.051$), and participants with low baseline serum CRP demonstrated a small increase at end-of-study ($P = 0.01$). No evidence was found of a correlation between the end-of-study cherry ACN exposure and any end-of-study inflammatory biomarker (data not shown). When participants were stratified as overweight or obese, homocysteine significantly decreased in overweight ($P = 0.041$) but not in obese men ($P = 0.506$). None of the other inflammatory biomarkers changed in either weight group (data not shown).

3.6. *Sensitivity analyses*

Sensitivity analyses that excluded participants who reported a greater than 1000 kcal/day difference in total energy intake between baseline and end-of-study did not alter the direction of any of the associations stated earlier (data not shown).

4. Discussion

This pilot study provides evidence of good tolerance and compliance to daily consumption of three cups of sweet cherries.

Table 3 – Comparison of urinary and serum biomarker levels at baseline and end-of-study, total and stratified by above or below the median baseline levels ($n = 37$), median (25th, 75th percentiles) and P-value.

Biomarker values at baseline	Baseline	End-of-study	P-value[a]
Urinary thromboxane B2	0.19 (0.15, 0.21)	0.19 (0.16, 0.24)	0.821
≤0.186 ng/mL	0.15 (0.14, 0.16)	0.16 (0.13, 0.17)	0.051
>0.186 ng/mL	0.22 (0.20, 0.29)	0.24 (0.22, 0.30)	0.112
Urinary prostaglandin E2 metabolite	131.4 (74.8, 290.3)	130.9 (88.9, 215.5)	0.268
≤131.4 pg/mL	74.8 (53.4, 109.4)	88.9 (69.8, 97.5)	0.355
>131.4 pg/mL	303.2 (222.8, 415.2)	220.1(177.7, 381.5)	0.071
Serum C-reactive protein	1.54 (0.92, 3.63)	2.20 (1.10, 3.31)	0.411
≤1.541 mg/L	0.92 (0.61, 1.48)	1.10 (0.48–1.59)	0.006
>1.541 mg/L	3.64 (2.48, 6.59)	3.40 (2.89, 7.69)	0.500
Serum homocysteine	10.2 (8.6, 11.6)	9.5 (8.7, 12.0)	0.531
≤10.20 µmol/L	8.6 (7.8, 9.8)	8.7 (7.9, 9.0)	0.644
>10.20 µmol/L	11.8 (11.3, 12.6)	12.1 (10.5, 13.5)	0.695

[a] P-value as determined by Wilcoxon signed-rank test.

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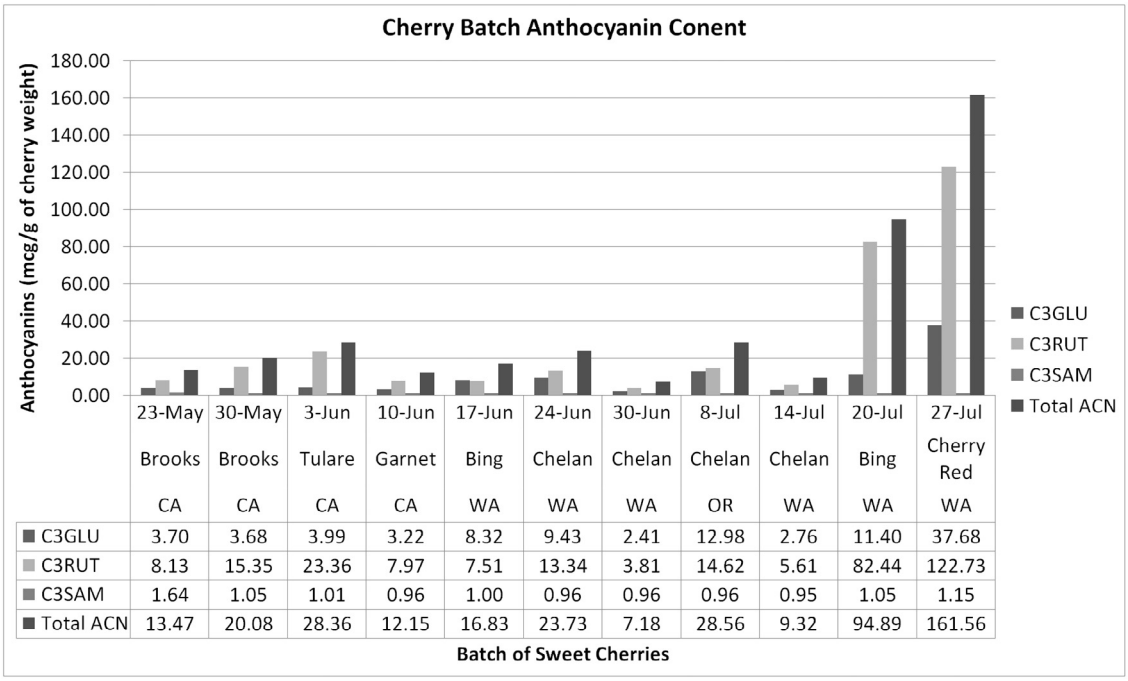

	23-May Brooks CA	30-May Brooks CA	3-Jun Tulare CA	10-Jun Garnet CA	17-Jun Bing WA	24-Jun Chelan WA	30-Jun Chelan WA	8-Jul Chelan OR	14-Jul Chelan WA	20-Jul Bing WA	27-Jul Cherry Red WA
C3GLU	3.70	3.68	3.99	3.22	8.32	9.43	2.41	12.98	2.76	11.40	37.68
C3RUT	8.13	15.35	23.36	7.97	7.51	13.34	3.81	14.62	5.61	82.44	122.73
C3SAM	1.64	1.05	1.01	0.96	1.00	0.96	0.96	0.96	0.95	1.05	1.15
Total ACN	13.47	20.08	28.36	12.15	16.83	23.73	7.18	28.56	9.32	94.89	161.56

Fig. 2 – Anthocyanin content of cherries, by batch. Concentration of anthocyanins (ACN; in mcg/g) significantly increased over time and differed by batch of cherries received. The following ACNs were quantified using liquid chromatography paired with tandem mass spectrophotometry (LC–MS/MS): cyanidin 3-glucoside (C3GLU); cyanidin 3-rutinoside (C3RUT); cyanidin 3-sambubioside (C3SAM). Total ACN is the sum of C3GLU, C3RUT, and C3SAM. Cherries were received at the specified dates in 2011 from either California (CA), Washington state (WA) or Oregon (OR). Varietals of cherries included Brooks, Tulare, Garnet, Bing, Chelan and Cherry Red.

These results suggest regular intake at these levels can be achieved and recommended if health benefits were shown. In this study of older, overweight or obese men, daily cherry intake resulted in significant increases in the concentrations of circulating (C3RUT in plasma) and excreted (C3GLU and C3RUT in 24-h urinary output) ACNs. Notably, we observed wide inter-individual variability in urinary and plasma ACN and PCA levels at end-of-study, perhaps reflecting metabolic differences between individuals as the differences between men did not correlate with measured end-of-study cherry ACN exposure levels.

In this study, improvement in inflammatory biomarkers was not demonstrated. Although our results contradict some earlier

Table 4 – Comparison of plasma and excreted anthocyanin levels detected versus below the limit of detection ($n = 30$), median (25th, 75th percentiles).

Anthocyanin	Plasma anthocyanin (ng/mL)			Urine anthocyanin (ng/mL)		
	Baseline	End-of-study	P-value[a]	Baseline	End-of-study	P-value[a]
C3GLU[b]	0.86 (0.00, 1.86)	0.00 (0.00, 0.00)	0.006	3.75 (0.00, 6.89)	6.87 (0.00, 12.11)	0.250
C3RUT[c]	0.00 (0.00, 0.00)	2.13 (1.37, 3.06)	<0.001	0.00 (0.00, 0.00)	64.16 (52.86, 91.16)	<0.001
C3SAM[d]	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	1.000	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.375
PCA[e]	0.00 (0.00, 0.00)	0.00 (0.00, 0.58)	0.289	13.55 (6.34, 26.40)	9.69 (6.96, 14.66)	1.000

Note: 0.00 indicates a value below the limit of detection.

[a] P-value as determined by McNemar's test on the presence (any value above the limit of detection) or absence (any value below the limit of detection) of each anthocyanin as follows: plasma C3GLU (16 present and 14 absent at baseline versus 6 present and 24 absent at end-of-study); plasma C3RUT (3 present and 27 absent at baseline versus 25 present and 5 absent at end-of-study); plasma C3SAM (4 present and 26 absent at baseline versus 3 present and 27 absent at end-of-study); plasma PCA (12 present and 18 absent at baseline versus 8 present and 22 absent at end-of-study); urinary C3GLU (17 present and 13 absent at baseline versus 20 present and 10 absent at end-of-study); urinary C3RUT (2 present and 28 absent at baseline versus all present and none absent at end-of-study); urinary C3SAM (7 present and 23 absent at baseline versus 4 present and 26 absent at end-of-study); urinary PCA (29 present and 1 absent at baseline versus all present and none absent at end-of-study).

[b] Cyanidin 3-glucoside (C3GLU).

[c] Cyanidin 3-rutinoside (C3RUT).

[d] Cyanidin 3-sambubioside (C3SAM).

[e] Protocatechuic acid (PCA).

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reports of an anti-inflammatory effect of sweet cherries (Bowtell, Sumners, Dyer, Fox, & Mileva, 2011; Jacob et al., 2003; Kelley et al., 2013), our observations are likely a result of a lower than expected ACN exposure from the cherries. In the present study, the per batch mean ± standard deviation concentration of C3RUT was 2.77 ± 3.85 mg/day. This was significantly lower than what was reported by Kelley et al. (2006) where the C3RUT concentration was determined to be 33.1 mg/day in Bing cherries with which a decrease in inflammatory biomarkers was observed.

Our results may indicate a higher level of ACN exposure is needed to observe anti-inflammatory effects in overweight/obese men. Alternatively, the somewhat unexpected low levels of inflammation in our population at baseline and the potential influence of a healthy participant bias in the study, despite the men being overweight or obese, may have limited any beneficial effects of cherries. Although we explored effects of cherry intake in those men with higher baseline inflammatory status, we were underpowered to detect these changes and did not observe any significant decreases in inflammation in these men. Rather, we observed a statistically but not clinically meaningful (Ridker, 2003) increase in CRP among participants with lower baseline CRP levels. Lastly, when men were stratified by overweight versus obesity, we observed a significant decrease in homocysteine after intervention only among overweight men. This finding supports the notion that men with more body mass may require a higher dose of cherry ACN to observe beneficial effects.

One of the more important findings of this work was the identification of wide variability in ACN content of the cherries from batch to batch and across cultivars from the same growing region and season. This is a relevant limitation to whole foods research. While ACN exposure from the cherries was not significantly associated with biomarkers of inflammation or measured ACN exposure in this study, our results suggest all subjects experienced a significant increase in circulating and excreted ACN in response to sweet cherry feeding. This response may be the results of an ACN threshold, as opposed to a linear dose, effect.

Homocysteine was investigated as a safety end-point given a single rat study suggesting a possible relationship with ACN intake (Nakagawa et al., 2002). The intervention did not alter serum homocysteine levels. Four participants demonstrated a rise in circulating homocysteine; however, the increase was associated with the discontinuation of folic acid supplementation during the trial (Wald et al., 2001).

Decreased dietary intake while on intervention was reported by most participants, with particular decreases noted in meal replacements, milk, and dairy-like beverages. Although this may have confounded our results, sensitivity analyses did not support any effect of change in calorie reporting on our results. This change in diet may be the result of greater awareness of foods among the male participants while on study, a possible role of cherries in satiety (Flood-Obbagy & Rolls, 2009), or more likely measurement error common to repeat assessments of diet using the same instrument in a small study (Goldbohm et al., 1995), especially given the stability of weight/BMI and the small increase in waist circumference over the study period.

Our study has a number of strengths including the use of the whole cherry fruit (as they are commonly consumed), 24-h urinary collections, measurement of ACN exposure, measurement of ACN per batch of cherries to assess actual exposure, and the use of multiple questionnaires to quantify the effects of cherry feeding on the participants' lifestyle (bowel habits and quality of life). Limitations to the study include the small sample size, self-reported dietary intake and related concerns with non-cherry dietary measurement error. Future efforts should include more participants, and should deliver a more consistent ACN exposure to reduce the wide inter-individual variability in ACN exposure. Other preferable options include freezing and blending cherries to use for delivery of an "average" ACN-density to all participants.

5. Conclusions

This pilot study provides evidence that 4 weeks of three cups/day of sweet cherry intake modifies human ACN levels and is well tolerated. Larger studies, with more precise ACN exposures in individuals with inflammation should be undertaken before ACN exposure via sweet cherries can be described as a functional food that clearly supports improvements in human health.

Conflict of interest

Dr. Thomson provided consultation on scientific strategic plan in 2010 for the Cherry Advisory Board & Washington State Fruit Commission. The other authors have no conflicts of interest to declare.

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