

**The effect of habitual consumption of anthocyanin-rich foodstuffs on
cardiovascular health in at-risk individuals**

by

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ABSTRACT

The Effect of Habitual Consumption of Anthocyanin-Rich Foodstuffs on Cardiovascular Health in At-Risk Individuals

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This thesis is an investigation of the ability of anthocyanin-rich purple carrots to attenuate cardiovascular disease risk factors through dietary intervention. By targeting foods that are already popular staples in the North American diet, purple carrots provide a realistic method of incorporating prevention or therapy into the average person's daily food intake. In the present research, 18 participants were screened for CVD risk and randomly assigned to either the Purple Carrot Group or Orange Carrot group. For the duration of the 12 week study period, participants had consumed 100g of their assigned color carrot, 2x /day for a total of 200g/d. Measurements were taken to assess various CVD risk factors at time points Week 0, 6, and 12. In conclusion, the findings suggest that habitual consumption of anthocyanin-rich purple carrots may improve serum triglyceride levels, and systolic and diastolic blood pressure in individuals at risk for cardiovascular disease.

A'udhu Billahi min ash-shaytaan-i'r rajeem. Bismillah-i'r Rahman-i'r Raheem

This work is dedicated to my ever loving and supporting Mommy and Baba, my brother Rakin, my sister Sabiha, and my brilliantly radiant wife Asra.

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Introduction

Research Objectives

The goal of the current research was to investigate whether replacing orange cultivars of carrots with purple cultivars of carrots would improve biomarkers for cardiovascular disease (CVD) in at-risk individuals following long-term habitual consumption.

It is hypothesized by the researcher that participants who consumed purple cultivars of carrots would have a greater improvement in CVD risk factors than participants who consumed orange cultivars of carrots, given the evidence showing the beneficial effects of anthocyanin-rich foodstuffs on biomarkers for cardiovascular disease risk and the high concentration of anthocyanins in purple carrots.

Thesis Organization

This thesis follows a manuscript style format and contains two chapters:

Chapter 1. The Role of Anthocyanins in Cardiovascular Health

This chapter provides a literature review of anthocyanins role in mediating cardiovascular disease risk. Included in the chapter is a description of the absorption and metabolism of anthocyanins, the physiological importance of blood pressure, inflammation and blood lipid levels on cardiovascular health, and an examination into the effect anthocyanin consumption has on these factors.

Chapter 2. An Investigation on the Effect of 12 Week Habitual Consumption of Orange and Purple Carrots on Cardiovascular Disease Risk Factors in Humans

This chapter presents the findings of a clinical trial that was conducted to assess the effectiveness of long-term orange and purple carrot consumption at improving biomarkers for CVD risk in participants who are already at risk.

CHAPTER 1: LITERATURE REVIEW

Cardiovascular Disease

It is well documented that cardiovascular disease (CVD) is amongst the world's leading cause of death from a non-communicable disease (1, 2) and is characterized by disorders of the heart itself and blood vessels (3). In Canada, approximately 1.6 million people are living with some form of CVD (4). As the current rates CVD risk factors such as obesity, hypertension, diabetes, elevated lipid levels, and chronic inflammation (3, 5), continue to increase (6), CVD will continue to contribute to high health and economic costs.

Most incidences of CVDs can be prevented by addressing behavioural risk factors such as tobacco use, unhealthy habitual diet, obesity, and sedentary lifestyle, all of which account for approximately 80% of CVD incidence. Such behaviours give rise to intermediate risk factors including high blood pressure, high blood sugar and raised lipid levels (5). Conversely, it has been shown that consumption of fruits and vegetables is inversely associated with risk of CVD (7), likely due to the profusion and variety of bioactive compounds present in these foodstuffs (3).

Researchers have examined the correlation between intake of fruits and vegetables and the risk of CVD. There have been numerous epidemiologic studies done that indicate a significant inverse correlation between consumption of fruits and vegetables and incidence of CVD. For example, in the Framingham Heart Study (8), a significant inverse association was observed between total vegetable and fruit consumption, as assessed by a single 24-hour recall questionnaire, and occurrence of ischemic stroke among 832 men. Moreover, the Nurses' Health Study and the Health Professionals' Follow-up Study (9) indicated that subjects with the highest fruit and vegetable intake (≥ 8 servings/day) had a relative risk of 0.8 compared to those consuming less than 3 servings/day of fruit and vegetables (9). An

increase in 1 serving/d was correlated with a 4% reduction in coronary heart disease risk. The investigators also found a significant inverse association between death from coronary heart disease and flavonoid intake from major food sources including apples, berries, and onions (9). Similar results were observed in the Physician's Health Study (10) and the Women's Health Study (11). Likewise, Knekt et al. (12) conducted a trial of subjects with a diagnosis of coronary heart disease that showed a Mediterranean diet rich of fruits and vegetables, α -linolenic acid significantly decreased recurrence of coronary heart disease events for up to 4 years compared with a regular low-fat diet.

The benefits of fruit and vegetable consumption have also been reported in association with the risk of stroke (8, 13, 14). Bazzano et al. (13) found a negative correlation between frequency of fruit and vegetable consumption and stroke incidence, stroke mortality, and CVD mortality. The incidence of stroke was significantly lower in participants who had consumed ≥ 3 servings/day of fruits and vegetables, compared with the reference group of < 1 serving/day. Furthermore, stroke mortality was greatly reduced in the highest quintile of consumption compared to the lowest (14). In a study examining the protective effect of fruits and vegetables, in particular cruciferous and green leafy vegetables, and citrus fruits and fruit juices, a negative correlation between intake and ischemic stroke risk was reported (15). Similar results were observed in the Health Professional Follow-up Study (16). Another study reported that intake of 5 servings of fruits and vegetables/day was associated with a 12% lower risk of CVD after considering standard cardiovascular risk factors (17). Some researchers, however, have not found an inverse association between fruit and vegetable intake and incidence of coronary heart disease (18). A possible reason could be due to the difficulty in attaining accurate self-reported fruit and vegetable consumption data (18). In addition, the effect of other underlying factors such as lifestyle, exercise, and socioeconomical status that are associated with increased fruit and

vegetable consumption (171) was not explored in these studies and may play a part in the conflicting results.

Numerous studies have suggested that the protective and antioxidant properties of vegetables are linked to the decreased incidence and risk of CVD (19). However, data linking a particular plant nutrient, such as antioxidant vitamins, to the observed cardioprotection, has been inconclusive (20, 21). Therefore, there is a possibility that researchers had overlooked non-nutritional components of plants that may be responsible for the plants cardioprotective effects (22). Subsequently, recent evidence shows that plant anthocyanins may have strong potential as cardioprotective agents (23).

Anthocyanins

Anthocyanins (anthos = flower and ky'anos = blue in Greek) belong to the flavanoid group of polyphenolic compounds and are the largest naturally occurring water-soluble pigments (24). They are distinct from other flavanoids because of their ability to form flavylium cations due to the anthocyanidin structure (25). Anthocyanins are responsible for the bright red-orange to blue-violet colors found in fruits and vegetables. Major sources of anthocyanins are fruit berries, cherries, black currents, red grapes, and red wine; key components of the human diet (3, 26).

In plants, anthocyanins naturally exist as glycosides consisting of an anthocyanidin covalently bonded to a sugar(29). Chemically, anthocyanidins are the polyhydroxylated or polymethoxylated derivatives of 2-phenylbenzopyrylium (30) which contains two benzoyl rings separated by a heterocyclic ring (29). Anthocyanidins are rarely found in nature due to their inherent instability (28). Glycosylation of the anthocyanidin provides enhanced stability and water solubility (31).

Over 400 different anthocyanins have been identified in nature (32). Each anthocyanin is differentiated by the specific sugar molecule bonded to the anthocyanidin and by the specific anthocyanidin attached. The differences between individual anthocyanidins are related to their structure (33) and include;

- The position and the number of hydroxyl groups on the aglycone
- The degree of methylation of the hydroxyl groups
- The position, number, and nature of attached sugars
- The number and nature of aliphatic or aromatic acids attached

These structural differences as well the eight conjugated double bonds which act as chromophores, the pH and temperature, and the presence of any co-pigments contribute to the color differences between anthocyanin-rich plant materials (30).

As of 2003, seventeen naturally occurring anthocyanidins had been identified (25). Of the seventeen, six anthocyanidins are widely distributed, accounting for over 90% of those found. These include cyanidin (Cy), delphinidin (Dp), pelargonidin (Pg), peonidin (Pn), malvidin (Mv), and petunidin (Pt) (29). The most common anthocyanins are the ones containing the three non-methylated anthocyanidins (Cy, Dp, and Pg) and are found in 80% of pigmented leaves, 50% of flowers and 69% of fruits (24). Cyanidin itself accounts for 50% of all anthocyanidins while 3-glucosides are the most common class of anthocyanidin glycoside. Therefore, the most prevalent anthocyanin in nature is cyanidin 3-glucoside (C3G) (24).

Anthocyanin structure is sensitive to changes in pH. They are most stable when the pH is between 1-3 where they form a flavylum cation and are red in colour. Anthocyanins form a colorless carbinol

pseudobase at pH 5 which is a less stable form and readily converts to a chalcone. At pH 7-8, the anthocyanin forms a quinoidal base which is blue-purple in colour (26).

Estimates of normal anthocyanin intake in humans vary greatly, ranging from a few to several hundred mg/day (26). In 1976 daily anthocyanin consumption was estimated to be 200 mg in the American population (32). Later it was determined that this was a gross over-estimate due to inaccurate food intake data as the average intake of anthocyanin in American adults in 2006 was estimated to be 12.5 mg/day (33). In European countries, the intake seemed to vary depending on the country. In Bilthoven, Netherlands, men typically consumed 19.8 mg/d while total mean intake in men from Turin, Italy was 64.9 mg/day. Anthocyanin consumption in women from Grenada, Spain was determined to be 18.4 mg/day whereas women from Turin, Italy had an average intake of 44.1 mg/day (34). The higher intake observed in Italians could be the consequence of their Mediterranean diet which is typically rich in berries, red and blue coloured fruits, and red wine (27).

The food industry is now very interested in anthocyanins as natural alternatives to synthetic dyes. This property, combined with the wide range of health promoting effects linked to anthocyanin consumption, has also increased the interest in using anthocyanin as functional food ingredients to prevent or treat chronic disease (27).

Absorption, Metabolism, Distribution, and Secretion of Anthocyanins

The daily consumption of anthocyanins is higher than the intake of other flavonoids found in the human diet such as genistein and quercetin (35). However the information regarding the absorption, metabolism and excretion of anthocyanins in humans (36) is more limited. Up until recently, studies seemed to indicate that anthocyanins were not absorbed and poorly metabolized compared to other

flavonoids, with less than 1% of ingested anthocyanins reaching circulation (35, 36). Recent studies have now revealed that bioavailability of anthocyanins may have been underestimated because the majority of anthocyanin metabolites have not yet been identified (35).

In both animal and human studies, ingested anthocyanins have been detected in circulation and in urine as their glycosylated forms, as well as methylated, glucuronide and/or sulfoconjugated derivatives (37- 43). Anthocyanins are some of the few plant derived polyphenols that are able to be identified in the plasma in their parent forms (3). Parent anthocyanin and anthocyanin derivatives persist in urine for 24h retaining their basic structure (25, 3). This was demonstrated in a study by Kay et al. (44) where two healthy volunteers consumed 20g of chokeberry extract containing 1.3g cyanidin 3-glycosides and had urine samples collected. Following consumption, the average urinary concentration of anthocyanins and their metabolites was 17.9 nM (range 14.9-20.9 nM) 5h post consumption and was still 12.1 pM (range 11.1-13.0 pM) in the 24h urine sample. The cumulative serum level total was 591.7 nM (range 197.3-986.1 nM) 2h post-consumption (44).

Moreover, anthocyanins and their metabolites are also prominent in early plasma concentrations (0-5h) post consumption depending on the individual compound and food matrix (3). Over time, the process of methylation occurs (6-24 h) suggesting that anthocyanin bioactivity is likely altered due to the metabolic transformation post-consumption (25). Studies assessing the concentrations of anthocyanins absorbed from food consumption were usually reported to be in the range of nM to low μ M (37, 39, 44-48). In a human study conducted by Kay et al. (46), the total concentration of parent anthocyanins and metabolites found in the serum over a 7 hour period following consumption was $172.96 \pm 7.44 \mu\text{g}\cdot\text{h}/\text{mL}$ and had a maximum serum concentration of $44.86 \pm$

2.82 µg/mL happening within 2.8 hours. Of the detected serum anthocyanins, 32.7% was the parent anthocyanin.

The current literature indicates that the majority of anthocyanin absorption occurs in the stomach (48, 52, 53) and small intestine (40, 42, 44, 46, 49). Although absorption of nutrients in the stomach is unusual, it has been established in rat studies where *in situ* gastric administration of anthocyanin glycosylates led to their detection in portal and systemic circulation (45, 49). This finding was further supported when rats fed black raspberries had absorbed up to 7.5% of the ingested anthocyanins into gastrointestinal tissues, a percentage that was much higher than the reported bioavailability of anthocyanins based on plasma and urine concentrations (50). This finding may suggest that uptake of anthocyanins into the gastric tissue may ably occur, but not transported into circulation as efficiently. Another *in situ* perfusion study in rats demonstrated that absorption of anthocyanins across the small intestine was highest in the jejunum tissue at 55.3% the ingested anthocyanins, followed by 10.4% in the duodenal tissue and no absorption occurring at the ileum (49, 51). These rat model studies indicate that anthocyanin glycosides can be absorbed into gastric tissues; however there is currently no direct evidence for this taking place in humans.

Anthocyanins are typically found and ingested as glycosides rather than as aglycones. As glycosides are hydrophilic, they would not be able to passively diffuse across biological membranes and thus would either need a specific active transport mechanism or would need to be hydrolyzed into the aglycone form to allow for passive diffusion and absorption (36). In addition, it has been speculated that non-glycosylated flavonoids are absorbed from the small intestine (52, 53) and this could also be true for the anthocyanins.

The distribution and accumulation of anthocyanins into body tissues following habitual consumption has been examined in pigs (56), rats (47, 57-60), and mice (61, 62). Venzo et al. (63) had reported that anthocyanins are rapidly moved from the blood into body tissues ($T_{1/2} = 22$ s). Talavera et al. (47) had found that following a 15 day blackberry-rich diet (14.8 nmol/kg), rats exhibited 605 nmol/g of anthocyanins and their derivatives in the jejunum, 68.6 nmol/g in the stomach tissue, 3.27nmol/g in the kidney, 0.38 nmol/g in the liver, and 0.25 nmol/g in the brain. The stomach tissue only contained parent anthocyanins (cyanidin-3-glucoside and cyanidin-3-pentoside) while the other organs had a mix of parent and conjugated metabolites, with the liver having the highest ratio of methylated cyanidins and the jejunum containing aglycones. Marczylo et al. (61) had recovered 700 nmol/g and 400 nmol/g of anthocyanins from mice gastrointestinal mucosa and kidneys after an oral dose of 500mg/kg cyanidin-3-glucoside.

Sakakibara et al. (62) have examined the accumulation of anthocyanins in the liver of mice after a 2 wk diet containing bilberry anthocyanins. Of the total anthocyanins taken up by the murine tissues, 51.5% was situated in the liver, suggesting that the liver is a main target for absorbed anthocyanins.

The kidneys also appear to be a major target organ for anthocyanin uptake. Vanzo et al. (59) reported that the total concentration of anthocyanins was double in the kidneys compared to the concentration in circulation and up to 4 times higher than the liver. This may signify that the kidney is more proficient in anthocyanin uptake in the short-term.

Removal of flavonoids, like anthocyanins, is done through the lungs, urine and feces. Respiratory metabolism has been demonstrated to be a key form of removal for a number of flavonoids. In Petrakis et al. (64), 11% of radiolabelled quercetin administered to a rat model was found in the lungs. Similarly,

Walle et al. (65) gave human subjects an oral dose of radiolabelled quercetin and determined that up to 52% was exhaled as $^{14}\text{CO}_2$. However, the respiratory removal of anthocyanins is still unknown (36).

Urinary excretion is the major form of excretion for flavonoids like anthocyanins. As such, excretion through urine is typically measured as an assessment of bioavailability (26). The majority of studies have reported low urinary excretion, varying from 0.004% to 0.1% of the intake (35, 26). However, Lapidot et al. (66) measured up to 5% of anthocyanin excretion following red wine consumption and Felgines et al. (42) reported 1.8% excretion in a strawberry feeding study. Of the anthocyanins excreted in the urine, only 32.5% were the parent glycoside while the remaining 67.5% were in the form conjugated metabolites (25). It has been reported that the maximal rate of urinary excretion takes place between 1 to 4 h and has an elimination half-life of 1.5 to 3 h (36).

Unabsorbed anthocyanins can also be egested through the biliary route (67), however to what extent this occurs in humans is still uncertain. Talavera et al. (49) found that anthocyanins and anthocyanin metabolites were detectable in rat bile 20 min after ingestion.

Anthocyanins and Blood Pressure

Studies conducted over the past decade have revealed the potential for anthocyanin-rich foods to affect cardiovascular structures and thus influence factors such as blood pressure. Hypertension is a risk factor for the development of cardiovascular disease as it correlated to reduced flexibility of arterial walls and improper blood flow (68).

A number of animal studies linked anthocyanin consumption to improved blood pressure. Studies involving consumption of anthocyanin-rich *Hibiscus sabdariffa* L. extract in rat models reported

decreased blood pressure in the intervention groups (69-72). These results were observed in rats with normal blood pressures, but the reduction was larger in hypertensive rats and a dose-dependent effect was observed in these studies particularly at low concentrations.

These results were replicated in rat models using various anthocyanin-rich foodstuffs such as purple carrot juice (73), red wine (74), bilberry (68), passion fruit peel (75), and grape powder (76). In Poudyal et al. (73), Wistar rats were fed a high fat and carbohydrate diet for 16 wks and were administered purple carrot juice for the final 8 wk. The results demonstrated that purple carrot consumption was able to attenuate increases in systolic blood pressure by 12% compared to the control. In addition, the purple carrot juice had significantly lower diastolic stiffness. These results may be attributed to the reduction in non-esterified fatty acids (NEFA) plasma concentrations observed following the purple carrot juice supplementation as NEFA inhibits aortic nitric oxide (NO) synthase activity in the endothelial. Thus decreasing NEFA can cause a vasorelaxant response (77, 78).

The findings from Andriambeloson et al. (74) also demonstrated a similar NO-dependent vasorelaxant effect in the aorta of male Wistar rats. The study investigated 10 fractions of red wine to determine which active constituents of red wine were responsible for vascular relaxation and found that only the anthocyanin and oligomeric-condensed tannins displayed the same activity as the original red wine. However the study found that delphinidin 3-glucosides, but not malvidin 3-glucosides or cyanidin 3-glucosides, were able to induce vasorelaxant activity.

Lewis et al. (75) further validated anthocyanins' capacity to affect hemodynamic parameters using passion fruit extracts. In this study, spontaneously hypertensive rats were used as the model as the breed experiences increases in blood pressure and heart rate as it ages. A single dose of either 2.5 or

50 mg/kg body weight of purple passion fruit peel extract was able to significantly reduce the blood pressure within 6 h of consumption and reduced the increase in blood pressure for 5 d post-consumption when compared to the control group. The study then evaluated the hypothesized active ingredients of purple passion fruit extract, edulilic acid, anthocyanin, and γ -aminobutyric acid. They found that 2.39 mg anthocyanin/kg body weight, the approximate amount found within 50 mg of purple passion fruit extract/kg body weight, decreased blood pressure to a greater extent than purple passion fruit extract, indicating that anthocyanins are one of the key components contributing to purple passion fruit peel extract's positive effect on blood pressure.

Studies involving anthocyanin-rich foods and hypertension in humans have yielded similar results to those in animal models (Table 1.1).

Table 1.1: Summary of human trials investigating the effects of anthocyanin-rich foodstuffs on blood pressure.

Author, Year	Participant Status	Foodstuff	Daily Dose	Study Duration	Results
Zibadi et al. (79)	Hypertensive	Passion fruit extract	400mg	4 wk	↓S-BP, ↓D-BP
Aviram et al. (80)	Hypertensive, taking ACE inhibitors	Pomegranate juice	50mL	2 wk	↓BP, ↓serum ACE activity
Aviram et al. (81)	Atherosclerotic patients w/carotid artery stenosis	Pomegranate juice	50mL	1 y	↓S-BP, ↓intima-media thickness
McAnulty et al (85)	Overweight	Blueberry powder	38g	6 wk	↓D-BP (in pre-hypertensive subjects)
Mozaffari-Khosravi et al. (86)	Type II Diabetes	Hibiscus tea	480mL	30 d	↓S-BP
McKay et al. (87)	Pre-hypertensive	Hibiscus tea	720mL	6 wk	↓S-BP
Hassellund et al. (88)	Hypertensive	Purified anthocyanins	640mg	4 wk	No effect on blood pressure
Wright et al. (89)	Pre-hypertensive	Purple carrot powder	Equivalent to 300g whole carrots	4 wk	No effect on blood pressure

Pomegranate juice consumption has been shown to have positive effects on blood pressure in two studies carried out by Aviram et al. (80, 81). In Aviram et al. (80), it was reported that serum ACE activity and blood pressure were significantly decreased by 36% and 5%, respectively. Although the

correlation between ACE activity and blood pressure has not been fully established, the authors suggested that there may be a potential relationship between the two based on their results. It has been noted that antioxidants, such as anthocyanins, can prevent reactive oxygen species (ROS)-induced endothelium contractions and increased vascular resistance (82). As ACE activity is linked to increased lipid peroxidation (83), an inhibition of ACE would further promote an antioxidant environment and induce a reduction in blood pressure. The reduction in systolic blood pressure following one year of juice consumption observed in (81) may be associated with the observed decrease in intima-media thickness in the study (84). Additionally, human studies have demonstrated that hibiscus tea consumption has the potential to reduce systolic blood pressure in mild hypertensive human participants (86,87). However, two studies published in 2013 found no effect of anthocyanin capsules and anthocyanin-rich purple carrot powder beverages on blood pressure.

Although the mode of action for the observed effect on blood pressure has not yet been fully confirmed, *in vitro* and *in vivo* studies may provide some insight into potential mechanisms. One possible mechanism is anthocyanin's activity as a vasorelaxant (25). In a study examining vasodilation capacity, phenolic content and antioxidant activity of various red wines, anthocyanins were reported to be the only phenol to be linked with vasodilation capacity (90). This was further confirmed by Andriambeloson et al. (74) where anthocyanin containing fractions of red wine demonstrated vasodilation whereas phenolic acid derivatives and flavanol classes did not bring about the same response.

Furthermore, anthocyanins may also prevent vasoconstriction which can lead to decreased blood pressure. Anthocyanins are powerful antioxidants and could prevent ROS and nitrogen species-induced endothelium contractions and re-establish proper endothelial function (80-82). Similarly

anthocyanins could inhibit ACE(80). ACE is involved in the production of angiotensin II (a strong vasoconstrictor) from angiotensin I, and inhibition of this enzyme would lead to vasorelaxation.

Anthocyanins and Inflammation

Inflammation is a normal response to protect the body against foreign pathogens or injury. However, complications can arise if this response occurs becomes chronic. Nuclear factor kappa B (NF- κ B) is a key transcription factor that regulates inflammation activity. An activated NF- κ B acts upon numerous genes to induce expression of pro-inflammatory cytokines, chemokines, adipokines, cell adhesion molecules, and acute phase proteins. These molecules also serve as quantifiable biomarkers and indicators of inflammation.

- Tumor Necrosis Factor alpha (TNF- α) – a pro-inflammatory cytokine that is mainly secreted by activated macrophages (91)
- Interleukin-6 (IL-6) – a pro-inflammatory cytokine that is mainly secreted by activated macrophages (92)
- Monocyte Chemoattractant Protein-1 (MCP-1) – a pro-inflammatory chemokine that plays a key role in the migration and infiltration of monocytes and macrophages (93)
- Soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1) – regulates the adhesion of monocytes and macrophages to the vascular endothelium (94)
- Soluble Intercellular Adhesion Molecule-1 (sICAM-1) – regulates the transmigration of monocytes and macrophages into tissues (94)
- High sensitivity C-Reactive Protein (hs-CRP) – a pro-inflammatory protein synthesized in the liver and facilitates the acute phase response (95)

An inflammatory response can be triggered by a great number of stimuli, including increased levels of ROS, fatty acids, and cytokines. When a stimulus triggers an inflammatory response, pro-inflammatory cells, such as macrophages and monocytes, migrate to the site and create ROS. These cells also secrete cytokines and chemokines through the action of NF- κ B to recruit more cells, produce more ROS and perpetuate the cycle of oxidative stress. If this cycle is allowed to continue unchecked, as is the case in obesity, then several chronic diseases may develop. Obese individuals are typically in a chronic inflammatory state. Increases in adipose tissue size influence the adipocytes to have pro-inflammatory secretions as a response to secreted TNF- α , IL-6, and MCP-1 from infiltrating macrophages that are recruited by the adipocytes. If this obesity-induced chronic inflammation is not treated, insulin resistance, type II diabetes, and CVD may develop (96).

Although anthocyanins can be potent antioxidants, it is becoming apparent to researchers that they exert their positive anti-inflammatory effects by altering several signalling pathways through activating transcription factors such as NF- κ B, affecting receptor activation, or having ligand activity (97, 98).

In vitro and *in vivo* animal model studies have been able to provide insight into the specific steps anthocyanins alter in cellular-signalling pathways that contribute to inflammation (99-102). Apo E/ mice fed an AIN-93G diet with blueberries for a 5 week period had reduced TNF- α and IL-6 serum levels as well as exhibited a decrease in TNF- α expression in the aorta. Furthermore, peritoneal macrophages had significantly decreased expression of TNF- α and IL-6 mRNA (99). In an animal model of metabolic syndrome (100), obese Zucker rats were fed a blueberry enriched diet for 8 weeks. Following the 8 week treatment, reductions in plasma TNF- α , IL-6, CRP were exhibited. The blueberry enriched diet also resulted in decreased liver expression of TNF- α , IL-6, and CRP, and NF- κ B, while TNF- α , IL-6 and NF- κ B

expression were reduced in adipose tissue. In a study by Kim et al. (101), lipopolysaccharide-stimulated inflammation Sprague-Dawley male rats were fed an atherogenic diet with 5% or 10% cranberry powder for 6 weeks; pro-inflammatory markers CRP and IL-6 were reduced while NO levels had increased. Moreover, a study by Qin et al. (102) observed anti-inflammatory effects following ingestion of an anthocyanin-rich foodstuff in an animal model, not only in the biomarkers but in gene expression as well. An insulin resistance-inducing, high fructose diet was fed to Wistar rats for 6 weeks. The drinking water given to the rats was unsupplemented (control) or supplemented with chokeberry extract to deliver either 100 or 200mg chokeberry extract/kg body weight. Rats given the enriched water had reduced TNF- α and IL-6 plasma levels. Furthermore, rats consuming the 200mg chokeberry extract/kg body weight had exhibited decreased gene expression of *Il6* and *Tnfa* in the adipose tissue.

The effect of anthocyanin-rich berries, either as whole fruits, freeze-dried powders, or extracts, has been extensively studied in human clinical trials. However, many of these studies have provided inconsistent results, both when these studies are segmented by the type of berry (ex; blueberry, cranberry, etc) and by the study group characteristics (obese, metabolic syndrome, etc).

The majority of the purported benefits of berry consumption in *in vitro* and *in vivo* animal studies have been demonstrated in short-term human trials involving acute inflammation (Table 1.2). In two different short-term studies, the effect of black currant extract powder (105) and juice (106) on acute inflammation was investigated. In an acute, exercise-induced inflammation model study from Lyall et al (105), 10 healthy subjects ingested two black currant extract powder capsules prior to and after a 30 minute bout of exercise and had blood samples taken before the exercise and 1, 2, and 24 hours after the exercise. The total anthocyanin content ingested from the supplementation regime was 240mg. Extract supplementation reduced TNF- α and IL-6 secretion and inhibited NF- κ B activation at the 24 hour

mark, when compared to the placebo (105). The second trial enrolled 20 healthy subjects in a randomized, cross-over study in which subjects consumed 250ml of 20% black currant juice (containing 50.5mg anthocyanins) or a control drink following a 72-hour diet devoid of flavonoids. 3 h after black currant juice consumption, there was no effect on acute vascular inflammation markers, sICAM-1 or sVCAM-1 (106). Although Jin et al. (106) had used healthy subjects as Lyall et al. (105) did; the two studies had contrasting results. As both study populations were not in a chronic inflammatory state and inflammation was not induced in Jin et al. (106) as it was Lyall et al. (105), it would reason that significant changes in inflammation markers would not be expected as quickly as 3 hours. Furthermore, the duration and dosage of black currant anthocyanins were quite different in the two studies. The juice trial was just a single dose containing 50.5mg anthocyanin while the extract powder trial was 2 doses, containing 120mg anthocyanin per dose.

Table 1.2: Summary of short-term human trials investigating the effects of anthocyanin-rich foodstuffs on inflammation biomarkers

Author, Year	Type of Inflammation	Participant Status	Foodstuff	Daily Dose	Anthocyanin Content	Study Duration	Results
Edirisinghe et al. (103)	Acute	Overweight	Strawberry powder	10g	81.6mg	6 h	↓ hs-CRP, ↓ IL-6
Sardo et al. (104)	Acute	Overweight	Black raspberry powder	45g		4 d	↓ IL-6
Lyall et al. (105)	Acute	Healthy	Black currant extract	2g	240mg	1 d	↓ TNF- α , ↓ IL-6, ↓ NF- κ B
Jin et al. (106)	Acute	Healthy	Black currant juice	250mL	50.5mg	3 h	No effect on sICAM-1 and sVCAM-1

Table 1.3: Summary of long-term human trials investigating the effects of anthocyanin-rich foodstuffs on inflammation biomarkers

Author, Year	Participant Status	Foodstuff	Daily Dose	Anthocyanin Content	Duration	Results
Reul et al. (107)	Obese male	Cranberry juice	125mL (week 1-4) 250mL (week 5-8) 500mL (week 9-12)	5.2mg (week 1-4) 10.4mg (week 5-8) 15.6mg (week 9-12)	12 wk	↓ sVCAM-1, ↓sICAM-1
Reul et al. (108)	Obese male	Cranberry juice	125mL (week 1-4) 250mL (week 5-8) 500mL (week 9-12)	5.2mg (week 1-4) 10.4mg (week 5-8) 15.6mg (week 9-12)	12 wk	↓ MMP-1
Basu et al. (109)	Metabolic Syndrome	Cranberry juice	480mL	24.8mg	8 wk	No effect on hs-CRP, IL-6
Dohadwala et al. (110)	CHD patients	Cranberry juice	480mL	94mg	4 wk	No effect on hs-CRP, sICAM-1
Lee et al. (111)	Type II diabetes	Cranberry powder	1.5g		12 wk	No effect on inflammation markers
Vidlar et al. (112)	Urinary tract infection	Cranberry powder	1.5g	1.65mg	24 wk	No effect on inflammation markers
Ellis et al. (113)	Overweight	Strawberry powder	10g	39mg	6 wk	No effect on hs-CRP or IL-6
Basu et al. (114)	Metabolic syndrome	Strawberry powder	50g	154mg	3 wk	No effect on hs-CRP
Basu et al. (115)	Metabolic syndrome	Strawberry powder	50g	154mg	8 wk	↓ sVCAM-1
Zunino et al. (116)	Obese	Strawberry powder	10g		3 wk	No effect on fibrinogen, IL-6, TNF- α , sICAM-1, sVCAM-1
Skoczyńska et al. (117)	Hypercholesterolemia	Chokeberry juice	250mL	19.13mg	6 wk	No effect on hs-CRP
Naruszewicz et al. (118)	Myocardial infarction patients taking statins	Chokeberry extract	255mg		6 wk	↓ hs-CRP, ↓IL-6, ↓ sVCAM-1, ↓sICAM-1, ↓MCP-1
Karlsen et al. (119)	Healthy	Purified anthocyanins	300mg		3 wk	No effect on hs-CRP, IL-6, TNF- α
Hassellund et al. (88)	Hypertension	Purified anthocyanins	640mg		4 wk	No effect on hs-CRP, IL-6, TNF- α
Karlsen et al. (120)	≤ 1 CVD risk factor	Bilberry juice	330mL		4 wk	↓ hs-CRP, ↓ IL-6
Lehtonen et al. (121)	Overweight/Obese	Bilberry fruit	100g		33-35 d	↓ TNF- α , ↓sVCAM-1
Kolehmainen et al. (122)	Metabolic syndrome	Bilberry fruit	400g	524mg	8 wk	↓ hs-CRP, ↓ IL-6
Basu et al. (123)	Metabolic syndrome	Blueberry powder	50g	742mg	8 wk	No effect on inflammation markers
Stull et al. (124)	Obese	Blueberry powder	50g	668mg	6 wk	No effect on inflammation markers
Riso et al. (126)	≤ 1 CVD risk factor	Blueberry powder	25g	400mg	6 wk	No effect on inflammation markers

Long-term studies (3-12 weeks) have also failed to demonstrate consistent anti-inflammatory effects post anthocyanin-rich berry consumption. Studies involving overweight and obese subjects had reported conflicting results, with some demonstrating a positive change in inflammation markers (107,108,121) and others showing no change (113,116,124). Similarly, there was no consistency in results among subjects with metabolic syndrome as the some studies showed decreases in sVCAM-1 (115), hs-CRP, and IL-6 (122), while some studies showed no effect (110,114,123). In addition, studies using whole-fruit or extracts of cranberries (107-112), strawberries (113-116), chokeberry (117,118) have yielded conflicting results on effects in altering inflammation status (Table 1.3). However, results from studies involving anthocyanin-rich bilberry have been promising as they have demonstrated decreasing in hs-CRP (120,122), IL-6 (120,122), TNF- α (121), and sVCAM-1 (121). Furthermore, these results have been reported in participants of various health statuses; subjects with \leq 1CVD risk factors (120), overweight and obese subjects (121), and subjects with metabolic syndrome (122).

Although not as extensively researched as berries, other anthocyanin-rich food stuffs are beginning to be investigated for their potential to exhibit anti-inflammatory activity. Purple carrots (*Daucus carota* L.) extracts potential effect in assuaging inflammation brought on by lipopolysaccharide stimulation was investigated using mouse RAW 264.7 macrophages and porcine aortic endothelial cells. The purple carrot extract had reduced macrophage mRNA expression of TNF- α and IL-6 in a dose-dependent manner and had decreased secretion of TNF- α and IL-6 from aortic endothelial cells (127). Purple potatoes had also been investigated for its effects on inflammation biomarkers in a human trial (128). 36 healthy men were randomized into 3 groups and consumed 150g of either white, yellow, or purple fleshed potatoes daily for 6 weeks. At the end of the trial, subjects who had consumed purple fleshed potatoes had significantly decreased hs-CRP and tended to have lower IL-6 plasma levels when

compared to the white fleshed potato group. No significant changes in TNF- α concentration were observed.

Although anthocyanins have been described as a powerful antioxidant, plasma concentrations of anthocyanin and its metabolites post anthocyanin-rich food consumption would suggest that direct ROS scavenging is unlikely. It has been hypothesized that anthocyanins may attenuate oxidative stress by activating Nrf2-regulated phase II enzymes (172). In the absence of oxidative stress, the transcription factor Nrf2 is sequestered in the cytoplasm by being bound the repressor protein Keap1. Once stimulated by oxidative stress, Nrf2 is released, moves into the nucleus and binds to antioxidant response element (ARE) consensus sequences. This results in the transcriptional modulation of a number of enzymes crucial in the cellular defence against ROS injury, including NAD(P)H: quinone oxidoreductase-1 (NQO-1) and heme oxygenase-1 (HO-1). The release of Nrf2 can also be stimulated by an inducer like anthocyanins. In an in vitro study by Speciale et al (172), human umbilical vein endothelial cells (HUVECs) treated with C3G had led to the activation of the Nrf2 pathway and increased expression of HO-1 and NQO-1 expression in a non-oxidative stress environment. Furthermore, this study also demonstrated that C3G treatment was able to counteract TNF- α induced nuclear translocation of NF- κ B in HUVECs. This may be a possible mechanism for the anti-inflammatory responses observed following consumption of anthocyanin-rich food stuffs.

Anthocyanins and Blood Lipids

It has been widely recognized that elevated serum triglyceride and LDL cholesterol concentrations, and hypercholesterolemia are significant risk factors for cardiovascular disease (CVD). This is due to their potential role in the development of atherosclerosis where the arterial walls thicken from fatty plaque accumulation, which can eventually lead to obstructed blood flow (129, 130).

Conversely, an elevated level of HDL-cholesterol is reported to be inversely associated with CVD risk (131). This positive attribute of HDL is due to its facilitation of reverse cholesterol transport in which cholesterol is transferred from circulation to the liver for bile excretion (132)

At its most promising, results from studies examining the effect of anthocyanin-rich foodstuffs on lipid profiles in animal models and humans demonstrated a reduction in total cholesterol, LDL cholesterol, and triglycerides with an increase in HDL cholesterol. Using 22 Sprague-Dawley rats, Yu et al. (133) found that C3G supplementation lowered serum lipid concentrations when rats were fed a high fat diet for 5 weeks to induce dyslipidemia and insulin resistance. After 5 wk of feeding this diet, 17 rats were randomized to receive the same diet plus either 100mg/kg body weight C3G, or saline via intragastric injection, for an additional 5 wk. Animals receiving the cyanidin-3-glucoside supplement had lower total cholesterol, LDL cholesterol, triglycerides and higher HDL cholesterol when compared to the saline supplemented group.

In 2013, two papers from Iran demonstrated the same positive effects of anthocyanin consumption on serum lipids in human trials. Kianbakht et al. (134) investigated the effect of hydroalcoholic extract of anthocyanin-rich whortleberry (*vaccinium arctostaphylos* L.) consumption in hyperlipidemic patients. Forty patients, aged 20-60, were supplemented for 2 months with three 350mg capsules/day of hydroalcoholic extract of anthocyanin-rich fruit whortleberry or placebo capsules.. Each extract capsule contained 2.45mg of anthocyanin for a daily total of 7.35mg anthocyanin. The study found that extract supplementation reduced triglycerides by 19.2%, total cholesterol by 27.6%, LDL cholesterol by 26.3%, and increased HDL cholesterol by 37.5% when compared to baseline levels. The second human trial, conducted by Asgary et al. (135) examined the effect of Cornelian cherry (*cornus mas* L.) supplementation on lipid profile in dyslipidemic youth. Forty dyslipidemic subjects, aged 9 to 16,

were randomized into two groups, one which received 50g of Cornelian cherry twice a day for 6 weeks and the other being the control group. The anthocyanin content of the Cornelian cherries provided to the subjects was not reported by the authors. Following the trial, the Cornelian cherry supplemented group had significant decreases in triglycerides (12.6%), total cholesterol (12.7%), and LDL cholesterol (10.0%) and had significant increases in HDL cholesterol (20.0%) when compared to baseline levels.

However, 24 studies from 2004 to 2014 in hamster (136), mouse (137-139), rat (140-144), and rabbit (145-147) models and human subjects (148-160), failed to replicate all of the benefits reported above; some replicated a few of the cardioprotective effects while others produced none. These studies used a variety of foodstuffs with different amounts of anthocyanins. Table 1.4 presents an overview of the reported outcomes with regards to lipid profiles in human trials.

In a study by Duthie et al. (148), 20 healthy, normotensive female subjects were randomly assigned to either a treatment group that consumed 750mL/day cranberry juice or a placebo group that consumed a drink with no anthocyanins for 2 weeks, in addition to their normal diet. The cranberry juice administered in the study contained 2.2mg anthocyanin per 750mL of juice and were predominately cyanidin and peonidin glycosides. No significant changes were observed in triglycerides, total cholesterol, and LDL- and HDL- cholesterol levels following the 2 week consumption of cranberry juice or placebo juice. Furthermore, there were no anthocyanins detected in blood or in urine samples, taken 12 hours post-juice consumption. Murkovic et al. (149) also found no effect of anthocyanin intake on lipid biomarkers associated with cardiovascular disease. In a 2 week, randomized, placebo-controlled study, 34 healthy subjects were given a standard diet and administered either 400mg spray-dried elderberry juice or a placebo. The elderberry juice capsules contained 100mg anthocyanin/capsule, verified by

HPLC. There were no significant differences in LDL- and HDL-cholesterol or triglycerides between the elderberry and placebo groups at the end of 2 wk trial.

However, it is important to note some key differences between these two studies when compared to the majority of studies that do show positive changes to the lipid profile following anthocyanin consumption. One key difference is the recruited subjects in these 4 studies. In the studies conducted by Kianbakht et al. (134) and Asgary et al. (135), all subjects were dyslipidemic when they had entered the study. In contrast, all subjects recruited in the trials by Duthie et al. (148) and Murkovic et al. (149) were healthy with normal lipid values at the beginning of the trial. The second major difference between these two groups of studies with differing results is the length of the study and anthocyanin dosage. The two studies which observed no effects were only 14 days in length while the other two studies which reported positive results were conducted for 42 and 60 days. The amount of anthocyanin used in these 4 studies also varied; 7.35mg/day in Kianbakht et al. (134), 2.2mg in Duthie et al. (148), and 100mg in Mukovic et al. (149). From this, it appears that length of habitual anthocyanin consumption may be a more important factor in influencing lipid levels than the amount of anthocyanins consumed daily.

A potential mechanism by which anthocyanin lowers serum triglycerides appears to involve the adenosine monophosphate-activated protein kinase phosphorylation (pAMPK)-lipoprotein lipase (LPL) signaling pathway, important in the regulation of triglycerides breakdown and thus in turn is important in the removal of serum triglycerides and fat metabolism in skeletal muscle and adipocytes. This hypothesis was investigated and confirmed by Wei et al. (161) using a KK-Ay mouse model and skeletal muscle cells and adipocytes *in vitro*. It was suggested that C3G, a common anthocyanin, had affected the activity of mRNA expression and protein expression of LPL. C3G supplementation had led to significant

increases compared to the control mouse group in plasma and skeletal muscle LPL activity (35% and 26%, respectively) but a decrease in the visceral adipose tissue (37.9%). This result was supported *in vitro* which demonstrated similar results in LPL mRNA and protein expression and a significant 2.2 fold increase in pAMPK expression in skeletal muscle and a significant 2 fold increase in visceral adipose tissue. Therefore, based on this finding it can be suggested that anthocyanin consumption decreases circulating triglycerides by increasing LPL activity, through the activation of pAMPK, in the blood and skeletal muscle, thus increasing the breakdown of triglycerides in the blood and their subsequent metabolic oxidation in the skeletal muscle.

Furthermore, Seymour et al. (162) have suggested another mode of action of lowering blood lipids through the activation of peroxisome proliferation-activated receptors (PPAR). PPAR is a transcription factor which targets genes involved in fatty acid oxidation, tissue lipolysis and fatty acid transport, principally expressed in the liver and skeletal muscles. PPAR agonists, such as fibrates, can also lower blood lipids and change fat metabolism in adipose tissues. Using a Dahl-Salt Sensitive rat model, which becomes insulin resistant and hyperlipidemic without becoming obese, the study found that 90 day supplementation of anthocyanin-rich tart cherry, plasma triglyceride and total cholesterol concentrations were lowered. Tart cherry supplementation had also significantly increased PPAR- α transcription level and PPAR- α target acyl-coenzyme A oxidase mRNA and activity. As tart cherry had similar effect on PPAR- α transcription levels as pharmacological PPAR- α agonist and had increased the expression and activity of acyl-coenzyme A oxidase,; anthocyanins may reduce plasma triglyceride and cholesterol levels through PPAR- α /acyl-coenzyme A oxidase associated pathways.

As previously noted, HDL cholesterol's role in reverse cholesterol transport helps reduce the risk for CVD. Cholesterol ester transfer protein (CETP), lecithin cholesterol acyltransferase (LCAT), and the

selective uptake by the liver of cholesteryl esters from HDL all effect efficacy of HDL in this process (132). A study conducted by Qin et al. (153) had investigated the effects of 12 week anthocyanin supplementation on cholesterol, CETP, and LCAT levels in 120 dyslipidemic subjects. The results of the trial had observed a correlation between plasma concentrations of HDL, LDL and CETP. In the anthocyanin group, the 13.7% increase in HDL cholesterol was negatively correlated to the 10.4% decrease in CETP mass following the 12 week supplementation period while the 13.6% decrease in LDL cholesterol was positively correlated with the decrease in CETP activity. In humans, the major site of CETP production occurs in hepatocytes. In an *in vitro* study reported in the same paper, human HepG2 hepatocytes were treated with C3G had exhibited significant reduction in CETP activity, in a dose-dependent response. Therefore it is suggested that the inhibition of CETP is a possible mechanism for elevating HDL cholesterol and decreasing LDL cholesterol.

Through research involving a variety of foodstuffs and with differing range of dosage and duration protocol in animal and human models, it has been observed that anthocyanin consumption may offer a cardioprotective effect by decreasing serum triglycerides, total cholesterol, and LDL cholesterol, and by elevating levels of HDL cholesterol. However, the number of studies that have reported all these outcomes is limited. Further research required in hyperlipidemic subjects with different dosing and treatment periods to determine the optimal daily anthocyanin consumption and minimum days of consumption needed for anthocyanins to exert its complete effect on blood lipids.

Table 1.4: Summary of human trials investigating the effects of anthocyanon-rich foodstuffs of blood lipid profiles

Author, Year	Type of Trial	Foodstuff	Daily Dose (Anthocyanin)	Duration	Triglyceride	Total Cholesterol	LDL	HDL
Duthie, 2006 (148)	Human	Cranberry Juice	2.2mg	14 days	No effect	No effect	No effect	No effect
Mukovic, 2004 (149)	Human	Elderberry juice powder	100mg	14 days	No effect	No effect	No effect	No effect
Gorinstein, 2006 (150)	Human	Red grapefruit	0.0515mg	30 days	Decrease	Decrease	Decrease	No effect
Zern, 2005 (151)	Human	Grape powder	27.72mg	28 days	Decrease	No effect	Decrease	No effect
Broncel, 2007 (152)	Human	Chokeberry	300mg	60 days	Decrease	Decrease	Decrease	No effect
Qin, 2009 (153)	Human	Berry-derived extracts	160mg	84 days	No effect	No effect	Decrease	Increase
Hassellund, 2013 (154)	Human	Purified anthocyanin	640mg	28 days	No effect	No effect	No effect	Increase
Kianbakht, 2014 (134)	Human	Whortleberry extract powder	7.35mg	60 days	Decrease	Decrease	Decrease	Increase
Soltani, 2014 (155)	Human	Whortleberry extract powder	90mg	28 days	Decrease	Decrease	Decrease	No effect
Lynn, 2014 (156)	Human	Tart cherry	273.5mg	42 days	Not measured	No effect	Not measured	No effect
Zhu, 2014 (157)	Human	Purified anthocyanin	320mg	168 days	Not measured	Not measured	Decrease	Increase
Broncel, 2010 (158)	Human	Chokeberry extract	213.3mg	60 days	Decrease	Decrease	Decrease	No effect
Asgary, 2013 (135)	Human	Cornelian cherry	Not reported	42 days	Decrease	Decrease	Decrease	Increase
Zhu, 2013 (15962)	Human	Purified anthocyanin	320mg	168 days	No effect	No effect	Decrease	Increase
Zhu, 2011 (160)	Human	Purified anthocyanin	320mg	84 days	No effect	No effect	Decrease	Increase

Anthocyanins and Metabolic Syndrome

Metabolic syndrome is a collective of related metabolic disorders, sometimes referred to as “The Deadly Quartet”, which include insulin resistance, dyslipidemia, hypertension, and abdominal obesity (166). As each of these disorders are a risk factor for CVD, and individual with metabolic syndrome is at considerable risk for a CVD event.

Evidence of anthocyanins’ ability to have positive effects when supplemented in a metabolic syndrome model was observed in two separate studies involving Wistar rats (73, 102). In Poudyal et al. (73), Wistar rats were fed a high-carbohydrate, high-fat diet for 8 weeks to induce a model of metabolic syndrome. After the 8 week diet, the rats were then supplemented for 8 weeks with either purple carrot juice or β -carotene. The purple carrot juice was able to attenuate the diet-induced glucose intolerance, dyslipidemia, hypertension, endothelial dysfunction and abdominal fat accumulation when compared to the β -carotene supplemented rats. As the administered purple carrot juice was low in β -carotene content, the investigators had attributed the metabolic syndrome reversing effects shown after juice consumption to anthocyanins. These results were further demonstrated in Qin et al. (102) in which a high fructose diet was fed to Wistar rats for 6 weeks to induce a model for metabolic syndrome. In addition to the diet, the drinking water given to the rats were enriched with 100 or 200mg chokeberry extract/kg body weight. Results of the study showed Rats that were given the chokeberry extract enriched water with the high fructose diet were able to attenuate epididymal fat accumulation, blood glucose, serum triglyceride, total cholesterol and LDL-cholesterol, TNF- α , and IL-6 increases when compared to the rats fed the high fructose diet without the enriched water. In addition, mRNA expressions in the insulin signalling pathway and glucose uptake in epididymal adipose tissue was up-regulated in chokeberry extract supplemented rats. Furthermore, rats consuming the 200mg chokeberry extract/kg body weight had exhibited decreased gene expression of *Il6* and *Tnfa* in the adipose tissue.

Clinical trials with human subjects with metabolic syndrome involving supplementation with anthocyanin-rich fruits have demonstrated positive results on some characteristics of metabolic syndrome. A trio of studies published between 2009-2011 by Basu et al. (114, 115, 109) reported varying levels of success of anthocyanin-rich berries in improving CVD risk in metabolic syndrome subjects. In a 2009 pilot study (114), 16 female subjects with metabolic syndrome who were given 50g/day of strawberry powder for a 3 week period observed significant decreases in total- and LDL-cholesterol but not in blood pressure, blood glucose, inflammation markers, or waist circumference. In a subsequent study (115), 27 metabolic syndrome subjects consumed 50g of freeze-dried strawberry powder for 8 weeks. This supplementation resulted in significant reductions in total- and LDL-cholesterol, and sVCAM-1, however similar to the 2009 study, did not illicit changes in HDL-cholesterol, triglycerides, blood glucose, blood pressure, or waist circumference when compared to the control. In a 2011 study conducted by the same researchers (109), subjects were supplemented daily with either 480mL of cranberry juice or a placebo drink for 8 weeks. Results from the study did not show significant alterations in lipid and glucose profiles, blood pressure, or inflammation markers hs-CRP or IL-6, when compared to the placebo group.

Two studies in 2010 and one in 2012 involving blueberry (123), black chokeberry (158) and bilberry supplementation (122) had reported differing effects. In the blueberry study (123), 48 metabolic syndrome subjects had consumed 50g of blueberry powder, which was equivalent to 350g of whole blueberries, for an 8 week trial. The results of the trial found that the habitual consumption of blueberries were able to improve only selected characteristics of metabolic syndrome, namely diastolic and systolic blood pressures compared to the water control group, but did not affect blood glucose, blood lipid profiles, or inflammation biomarkers. Supplementation with black chokeberry over the same

trial period was able to have a similar affect on blood pressure, and was able to improve lipid profile as well. In a study involving 25 subjects with metabolic syndrome investigated the effect of 8 week supplementation of 300mg/day of black chokeberry extract, significant reductions in systolic and diastolic blood pressure, total- and LDL-cholesterol, and serum triglyceride levels were observed when compared to baseline measurements (158). However like in blueberry supplementation, fasting blood glucose levels, hs-CRP, and waist circumference were not significantly changed. Although bilberry supplementation did not result in the observed effects on hypertension and dyslipidemia, it was able to reduce inflammation biomarkers hs-CRP and IL-6 following an 8 week, 400g fresh bilberries (1356mg anthocyanins/day) supplementation regime in 15 metabolic syndrome subjects (125).

Carrots and CVD Biomarkers

Carrots, one of the most consumed vegetables in North America, are a major source of fibre and carotenoids (164). Epidemiological studies describing the relationship between high fibre diets and CVD risk have shown to be negatively correlated (165) while studies investigating the relationship between carotenoid intake and CVD incidences are inconclusive (166). A clinical trial by Rapola et al. (167) had supplemented 1862 smokers with previous myocardial infarction with α -tocopherol, β -carotene, both, or a placebo reported no significant differences in the number of coronary events between any of the groups.

Human trials examining the effects of carrots on biomarkers of CVD risk have been few in numbers, typically short-term in duration, and have produced conflicting results regarding habitual carrot consumption on serum cholesterol levels. A study conducted by Robertson et al. (168) reported a significant 11% reduction in serum cholesterol and a 50% increase in fecal fat and bile excretion following a 3 wk, 200g/day carrot supplementation period. However, two 3 wk studies, one in which

participants were supplemented with 20g/day of concentrated dietary fibre from carrots (169) and second in which participants were supplemented with 405g and 688g of raw, frozen, blanched, or canned carrots (170), had reported no effect of carrot consumption on serum cholesterol levels.

Although carrots are typically found in markets in their orange varieties, ancient breeds of carrots are purple in color and rich in anthocyanins (164). Recently, *in vitro* (127), *in vivo* animal (73), and human (89) studies have investigated the effect of purple carrot on CVD biomarkers. In an *in vitro* study, the potential effect of purple carrot extracts in assuaging inflammation brought on by lipopolysaccharide stimulation was investigated using mouse RAW 264.7 macrophages and porcine aortic endothelial cells. The purple carrot extract had reduced macrophage mRNA expression of pro-inflammatory cytokines TNF- α and IL-6 in a dose-dependent manner and had decreased secretion of TNF- α and IL-6 from aortic endothelial cells (127). In an animal *in vivo* study using a high fat/high carbohydrate diet rat model, Poudyal et al. (73) had demonstrated that a purple carrot juice supplementation was able to moderate increases in blood pressure and lower left ventricle fibrosis compared to rats who did not receive purple carrot juice supplementation. This study also found that the supplemented rats had significantly less diastolic stiffness in comparison to the control group. Finally, in a human trial by Wright et al. (89) 16 male participants were supplemented for 4 weeks with either 24.9g/day of dried purple carrot powder, equivalent to 300g of fresh carrot and contained 118.5mg of anthocyanins, or 24.9g/day of dried orange carrot powder containing 0mg of anthocyanins. Following the trial period, no statistically significant changes were reported in either group for; body fat %, blood pressure, lipid levels, or C-reactive protein.

Future Directions

Following ingestion, anthocyanins can be biotransformed into conjugated metabolites which are likely excreted through urine, but they can be recycled back into circulation, or egested through the biliary route. It is a possibility that intact anthocyanins, their metabolic forms, and excretory products may become undetected when chemically bonded to other components such as protein or other components in bloodstream. A realistic solution for this limitation can be using radiolabeled (^{14}C or ^3H) tracer anthocyanins in animal and human studies to easily identify and characterize all major metabolites generated (3) *in vivo*. Currently, it appears that the reported literature research in the area of anthocyanin metabolism in the gut microflora is limited. Though it was seen in a bacterial preparation mimicking function of human microflora that the smaller phenolic derivatives demonstrated similar anti-inflammatory effects as the parent compound (3); and hence more studies are warranted to answer similar phenomenon as indicated above.

Furthermore, a long-term, randomized human trial is needed to assess where the effects of an anthocyanin-rich staple food is able to elicit similar cardioprotective activity as lesser consumed berries like chokeberries and bilberries. Carrots are among the most popular vegetables consumed in the North American diet, with an average per capita intake of 14 pounds (6.36kg) of carrots per year (19). As carrots are popular diet staples, it makes them prime candidates as functional foods, as people are already incorporating them into their diet, although they are most likely consumed in their orange forms. Ontario-grown, purple carrots would be a good candidate for research as they are inexpensive, have a long growing period, and can be incorporated into Canadian diets more easily than rare berries or anthocyanin extract supplements. Therefore, a study to elucidate whether replacing orange cultivars with purple cultivars of carrots would improve biomarkers of CVD in at-risk human subjects is necessary.

Conclusion

The current body of literature in *in vitro* and *in vivo* animal studies have consistently shown the effects of anthocyanin-rich foods and extracts on improvement in risk factors for CVD, namely in dyslipidemia, hyperglycemia, hypertension, and inflammation and have been able to propose potential mechanisms of actions for these activities. However, clinical trials have not observed the same effects in humans consistently. Human trials, although promising, have varied between reporting improvements in all, some, or none of the above mentioned CVD risk factors. This in large part may be attributed to the varying forms of supplementations, dosage of anthocyanins, treatment duration, and baseline characteristics of the human subjects.

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CHAPTER 2: AN INVESTIGATION ON THE EFFECT OF 12 WEEK HABITUAL CONSUMPTION OF ORANGE AND PURPLE CARROTS ON CARDIOVASCULAR DISEASE RISK FACTORS IN HUMANS

2.1 Introduction

It is well documented that cardiovascular disease (CVD) are amongst the world's leading cause of death from non-communicable diseases (1, 2) and is characterized by disorders of the heart itself and blood vessels (3). In Canada, approximately 1.6 million people are living with some form of CVD (4). As the current rates CVD risk factors such as obesity, hypertension, diabetes, elevated lipid levels, and chronic inflammation (3, 5), continue to increase (6), CVD will continue to be a substantial burden both in terms of human suffering and in economic costs.

Studies have linked increased consumption of fruits and vegetables with a reduction in CVD risk (7). These foodstuffs contain a large number of bioactive compounds including anthocyanins. Anthocyanins are the largest group of, and are at the highest concentrations, of the naturally occurring water-soluble pigments. They belong to the flavanoid group of polyphenolic compounds. Anthocyanins are responsible for the bright red-orange to blue-violet colors found in fruits and vegetables. As major sources of anthocyanins are fruit berries, cherries, black currants, red grapes, and red wine, they are key components of the human diet. (1-4). In plants, anthocyanins naturally exist as glycosides where an anthocyanidin is linked to a sugar molecule (4, Mazza and Miniati 1993). Studies revealed that cyanidin itself accounts for 50% of all anthocyanidins, while 3-glycosides are the most common class of anthocyanidin glycoside. Therefore, the most prevalent anthocyanin in nature is cyanidin 3-glucoside (1).

Recently, studies have shown that consumption of anthocyanin-rich foods can improve a number of risk factors of CVD such as atherosclerosis (8, 9, 10, 11), inflammation (10, 12, 13), lipid levels

(14, 15, 16), diabetes (17, 18) and may lower the risk of coronary heart disease (10, 19). Furthermore, epidemiological studies have suggested an inverse relationship between anthocyanin consumption and CVD risk.

Carrots are among the most popular vegetables consumed in the North American diet, with an average per capita intake of 14 pounds (6.36kg) of carrots per year (20). As carrots are popular dietary staples, it makes them prime functional food candidates. Though commonly consumed, carrots are most likely being ingested in their white and orange forms (56).

The current study focused on the ability of anthocyanin-rich purple carrots to attenuate cardiovascular disease risk factors through dietary intervention. By targeting foods that are already popular staples in the North American diet, purple carrots provide a realistic method of incorporating prevention or therapy into the average person's daily food intake.

2.2 Methods and study design

In the present study, 18 participants were screened for CVD risk and randomly assigned to one of two groups; Purple Carrot Group or Orange Carrot group. For the duration of the 12 week study period, participants had consumed 100g of their assigned color carrot, twice a day for a total of 200g/d. Measurements were taken to assess various CVD risk factors at time points Week 0, 6, and 12 at the University of Guelph's Human Nutraceutical Research Unit.

Screening (Participants selection)

Participants were screened for the following cardiovascular disease (CVD) risk factors; hypertension ($\geq 130/\geq 85$ mmHg); BMI > 27 , waist circumference > 88 cm (female) and > 102 cm (male), low HDL cholesterol (< 1.00 mmol/L for males and < 1.29 mmol/L for females), high LDL cholesterol (> 4.00 mmol/L), high fasting blood glucose (> 5.6 mmol/L), high serum triglycerides (> 17 mmol/L), and overt Diabetes Mellitus. Participants must have had at least 2 of the above CVD risk factors to be included in the study.

Participants were excluded if they had any of the following; active disease (peripheral vascular disease, degenerative kidney disease, degenerative liver disease, cancer, acid reflux disease, endocrine disorders (other than diabetes), currently smoking tobacco cigarettes, pregnancy, or a malabsorption disorder that could affect the bioavailability of anthocyanins.

Groups

Qualified participants were randomly assigned into one of two groups, Orange Carrot group or Purple Carrot group. Orange Carrot group participants consumed 100g of raw Ontario-grown orange carrots (grown at Muck Crops Research Station, University of Guelph) twice a day as part of their usual diet, for a 12-week period. Participants placed in the Purple Carrot group consumed 100g of raw Ontario-grown purple carrots (grown at Muck Crops Research Station, University of Guelph) twice a day for a 12 week period.

The Orange Carrot group had 9 participants (5 females and 4 males, mean age = 39.6 years) and the Purple Carrot group began with 9 participants (5 females and 4 males, mean age = 41.4 years) with one female participant dropping out at the week 7 mark.

Carrots

The orange carrot breed “Cellobunch” was provided to the Orange Carrot group while the purple carrot breed “Purple Sun” was provided to the Purple Carrot group. The Purple Sun carrots contained 3.5mg of the anthocyanin, cyaniding 3-glucoside (C3G) per gram of carrot. Thus participants in the Purple Carrot group consumed ~700mg C3G/d. The Cellobunch carrots contained no anthocyanins.

Diet and Exercise

All study participants were required to maintain their usual diet as much as possible while including the carrots. Prior to beginning the study, participants completed a 7-day diet record. Likewise, participants completed a 7-day diet record, in the same fashion as the baseline record, during the final week of the study to ensure diet consistency. Participants were required to maintain their usual level of activity or exercise regime throughout the duration of the study. The only lifestyle change of the participants was the incorporation of the carrots into their usual diet.

Medication Use

As eligible participants were at high risk of CVD at baseline, some participants were already on medication to manage their CVD risk. Participants included in the present study had stable medication

use for at least 3 months prior to the study and medication use was not changed for reasons other than the study's dietary intervention during the study duration.

Measurements

Measurements were taken to assess various cardiovascular risk factors such as obesity, blood pressure, insulin resistance and blood glucose, blood lipids, and inflammation at the University of Guelph's Human Nutraceutical Research Unit.

Body Measurements: On a weekly basis participants were measured for weight (kg), blood pressure (mmHg), and waist and hip circumference (cm) by the research group. In addition, bioelectrical Impedance Analysis (BIA) to assess body fat % was measured at week 0, week 6, and week 12 using a Bodystat 1500 machine to estimate body composition.

Fasting Blood Glucose: Fasting blood samples were collected in vacutainers – containing a clot activator - by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample were allowed to clot for 30 minutes at room temperature and then separated by centrifugation. Samples were then refrigerated at 2°C and sent to Lifelabs Medical Laboratory Services for measurement.

Triglyceride, LDL, HDL, and Total Cholesterol: Fasting blood samples were collected in vacutainers – containing a gel for serum separation - by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample was mixed by gentle inversion and were allowed to clot for 30 minutes at room temperature and then separated by centrifugation. Samples were then refrigerated at 2°C and sent to Lifelabs Medical Laboratory Services for measurement.

Fasting Insulin: Fasting blood samples were collected in vacutainers – containing a gel for serum separation - by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample was mixed by gentle inversion and were allowed to clot for 30 minutes at room temperature and then separated by centrifugation. The serum was transferred into labelled tubes and frozen at -20°C. The frozen serum samples were sent to Lifelabs Medical Laboratory Services for measurement.

C-Reactive Protein: Fasting blood samples were collected in vacutainers – containing a gel for serum separation - by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample was mixed by gentle inversion and were allowed to clot for 30 minutes at room temperature and then separated by centrifugation. Serum samples were then refrigerated at 2°C and sent to Lifelabs Medical Laboratory Services for measurement.

Hemoglobin A1C: Fasting blood samples were collected in EDTA vacutainers by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample was mixed by gentle inversion. Whole blood samples were then refrigerated at 2°C and sent to Lifelabs Medical Laboratory Services for measurement.

Fibrinogen: Fasting blood samples were collected in sodium citrate vacutainers by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample was mixed by gentle inversion and then separated by centrifugation. The plasma was transferred into labelled tubes and refrigerated at 2°C. The plasma samples and collection tubes were sent to Lifelabs Medical Laboratory Services for measurement.

Lipoprotein (a): Fasting blood samples were collected in vacutainers – containing a gel for serum separation - by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample was mixed by gentle inversion, were allowed to clot for 30 minutes at room

temperature and then separated by centrifugation. The serum was transferred into labelled tubes and frozen at -20°C. The frozen serum samples were sent to Lifelabs Medical Laboratory Services for measurement.

TNF-alpha and IL-6: Fasting blood samples were collected in vactainers – containing a clot activator - by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample was mixed by gentle inversion and were allowed to clot for 30 minutes at room temperature and then separated by centrifugation. The serum was transferred into tubes labeled TNF-alpha and IL-6 frozen at -80°C. Samples were later thawed at room temperature and measured using the appropriate ELISA assay (Thermo Scientific).

Phytonutrient Bioavailability and Metabolism: Fasting blood samples were collected in vactainers – containing sodium herapin - by phlebotomists at week 0 (baseline), at week 6 (midpoint) and week 12 (endpoint) of the study. Each sample was mixed by gentle inversion and were allowed to clot for 30 minutes at room temperature and then separated by centrifugation. The plasma was transferred into tubes containing 15µL formic acid and frozen at -80°C. Morning void urine samples were collected at week 0, week 6 and week 12 to assess differential carrot phytonutrient metabolism between participants. 1mg L-ascorbic acid per mL of sample was added to the urine and frozen at -80°C.

Blood and urine samples were later thawed at room temperature and treated with 40µL of an enzyme solution containing Apigenin and β-Glucuronidase. Following an incubation at 37°C for 18 h, 950µL of acidic methanol was added to the samples to complete the protein precipitation. The sample solution was then centrifuged at 20000xg for 5 minutes at 4°C and the supernatant was then filtered using a vacuum manifold. The samples were transferred into HPLC vials and analyzed using LC-3Q-MS to measure;

- Cyanidin 3-glucoside – the most prevalent anthocyanin in nature (57)

- Protocatechuic Acid – the major human metabolite of C3G (58)
- Chlorogenic Acid – the ester of caffeic acid and quinic acid with antioxidant properties and one of the most abundant polyphenols in fruits and vegetables. Chlorogenic acid is an intermediate in the synthesis of lignin (59)
- Caffeic Acid – one of the most common polyphenols found in plants. Caffeic acid is an intermediate in the synthesis of lignin and has antioxidant properties (59)
- Ferulic Acid – an abundant phenolic phytochemical with antioxidant properties found in plant cell walls and is covalently bonded to lignin (60).
- B-carotene – abundant in carrots and account for half of the provitamin A found in the human diet (61)

Statistical Analysis: A mixed ANOVA analysis was conducted using SPSS Statistical Package for the Social Sciences (SPSS v.21.0, IBM Corporation, Armonk, USA) to compare the mean differences of the measured CVD biomarkers between the two groups, using the within-subject factor of time (Week 0, 6, and 12) and the between-subject factor of carrot color (purple or orange). The sample size of 30 (15 participants to complete all 12 weeks in each group) was calculated to achieve 80% power to detect a 10% change in the measured biomarkers. A p value of ≤ 0.05 will be used as an indicator of a significant change.

2.3 Results

Table 2.1 shows the number of participants that had fit each of the pre-study inclusion criteria. Every study participant had met the waist circumference criteria. Nine participants had elevated systolic blood pressure and eight participants had entered the study with high diastolic blood pressure. There

was a misbalance in the number of participants with elevated serum triglycerides, with five in the purple carrot group but only one in the orange carrot group.

Table 2.1: The number of participants in each group, broken down by inclusion criteria.

	Orange Carrot Group	Purple Carrot Group
BMI	9	7
Waist Circumference	9	9
Systolic Blood Pressure	5	4
Diastolic Blood Pressure	5	3
Serum Triglycerides	1	5
LDL Cholesterol	1	1
HDL Cholesterol	3	2
Fasting Blood Glucose	1	3

Table 2.2 reports the findings of the Week 0, 6, and 12 body and blood measurements. As shown in the table, the Purple Carrot group Week 12 measures for systolic blood, diastolic blood pressure, and serum triglycerides were statistically significantly lower than their Week 0 measures by 5.2%, 7.4%, and 14.3%, respectively, following 12 weeks of habitual purple carrot consumption. However, the other measurements were not statistically different following the 12-week intervention. None of the Week 12 measures for the orange carrot group were statistically significantly different following 12-week habitual consumption of orange carrots.

Tables 2.3 and 2.4 report the plasma and urine concentrations for; chlorogenic acid, caffeic acid, ferulic acid, cyanidin 3-glucoside, protocatechuic acid, and carotenoids. The main effect of time showed a statistically significant difference in caffeic acid concentration in the plasma between Week 0 and Week 6; however this was not specific to either carrot group. There was also a statistically significant interaction between the intervention and time on ferulic acid concentrations in the plasma in both the orange and purple carrot group. In the urine, the main effect of time showed a statistically significant

difference in Cyanidin-3-Glucoside concentration in urine between Week 0 and Week 6, with the purple carrot group exhibiting a 258% increase. The orange carrot group had shown a 242% increase over the same time period. The main effect of time had also showed a statistically significant difference in protocatechuic acid concentration in urine between Week 0 and Week 12 for both groups, with the purple carrot group having a 37.82% reduction and the orange carrot group having a 29.25% reduction.

Table 2.2: Participant measurements taken at Week 0, Week 6, and Week 12 of the study trial and the ‘Trial Difference’ between Week 12 and Week 0 values. ^a denotes a statistically significant change between Week 0 and Week 12.

	Orange Carrot Group					Purple Carrot Group				
	Week 0	Week 6	Week 12	Trial Difference	Trial Difference %	Week 0	Week 6	Week 12	Trial Difference	Trial Difference %
BMI (kg/m²)	31.6	31.6	31.7	0.1	0.4%	29.6	29.5	29.6	-0.1	-0.3%
Waist Circumference (cm)	100.7	101.1	100.7	0.0	0.0%	97.3	97.0	97.5	0.2	0.2%
Systolic Blood Pressure (mmHg)	127.8	127.7	129.2	1.4	1.1%	137.0	128.6	129.9	-7.0 ^a	-5.2% ^a
Diastolic Blood Pressure (mmHg)	85.1	83.1	84.8	-0.3	-0.3%	84.5	81.6	78.2	-6.3 ^a	-7.4% ^a
Body Fat (%)	35.0	35.0	35.2	0.2	0.7%	33.1	32.2	32.8	-0.3	-0.9%
Triglycerides (mmol/L)	1.2	1.1	1.3	0.1	4.0%	2.0	1.7	1.7	-0.3 ^a	-14.3% ^a
Total Cholesterol (mmol/L)	5.2	5.0	5.0	-0.2	-3.8%	5.2	5.2	5.3	0.1	1.5%
LDL Cholesterol (mmol/L)	3.2	3.0	3.1	-0.1	-2.5%	3.1	3.3	3.4	0.3	8.0%
HDL Cholesterol (mmol/L)	1.5	1.4	1.3	-0.1	-9.6%	1.2	1.2	1.1	0.1	-2.5%
Lipoprotein (a) (mg/L)	509.3	524.7	560.6	51.2	10.0%	339.8	335.6	322.0	-17.8	-5.2%
Fasting Glucose (mmol/L)	5.2	5.7	5.3	0.1	1.0%	5.3	5.4	5.5	0.2	3.8%
Insulin (pmol/L)	102.3	72.3	85.0	-17.3	-16.9%	75.3	91.3	90.3	15.0	19.9%
HbA1c (%)	5.8	5.8	5.7	-0.1	-1.7%	6.0	6.0	5.8	-0.2	-3.3%
Fibrinogen (g/L)	2.7	2.9	2.8	0.2	6.4%	3.0	3.1	3.0	0.0	-1.7%
hsCRP (g/L)	3.6	4.2	4.5	0.8	23.1%	2.6	3.8	2.8	0.2	6.8%
TNF-a (pg/mL)	40.1	37.1	39.3	-0.8	-2.0%	41.8	36.8	47.8	6.0	14.5%
IL-6 (pg/mL)	6.6	6.4	6.2	-0.4	-5.3%	14.1	11.7	12.7	-1.4	-10.1%

Table 2.3: Plasma concentrations of chlorogenic acid, caffeic acid, ferulic acid, cyaniding 3-glucoside, protocatechuic acid, and carotenoids from participants' 12-hour blood samples taken at Week 0, Week 6, and Week 12 of the study trial, and the 'Trial Difference' between Week 12 and Week 0 values. ^a denotes a statistically significant change between Week 0 and Week 12. ^b denotes a statistically significant change between Week 0 and Week 6.

	Orange					Purple				
	Week 0	Week 6	Week 12	Trial Difference	Trial Difference %	Week 0	Week 6	Week 12	Trial Difference	Trial Difference %
Chlorogenic Acid (nM)	Not Detected	Not Detected	Not Detected	N/A	N/A	Not Detected	Not Detected	Not Detected	N/A	N/A
Caffeic Acid (nM)	5.9	21.6 ^b	12.2	6.3	106.8%	8.4	11.5 ^b	11.2	2.8	33.3%
Ferulic Acid (nM)	3.1	10.5	47.9	44.8 ^a	1445.2% ^a	3.7	28.3	22.1	18.4 ^a	497.3% ^a
Cyanidin-3-Glucoside (nM)	0.23	0.17	0.17	-0.06	-26.1%	0.33	0.62	0.62	0.29	87.9%
Protocatechuic Acid (nM)	62.0	60.5	81.8	19.8	31.9%	71.4	75.3	74.6	3.2	4.5%
Carotenoids (nM)	4235.6	4500.1	3201.9	-1033.7	-24.4%	3673.1	4496.4	4339.9	666.8	18.2%

Table 2.4: Urine concentrations of chlorogenic acid, caffeic acid, ferulic acid, cyaniding 3-glucoside, protocatechuic acid, and carotenoids from participants' morning urine sample taken at Week 0, Week 6, and Week 12 of the study trial, and the 'Trial Difference' between Week 12 and Week 0 values. ^a denotes a statistically significant change between Week 0 and Week 12. ^b denotes a statistically significant change between Week 0 and Week 6.

	Orange					Purple				
	Week 0	Week 6	Week 12	Trial Difference	Trial Difference %	Week 0	Week 6	Week 12	Trial Difference	Trial Difference %
Chlorogenic Acid (nM)	140	100	110	-30	-21.4%	25.9	27.6	27.6	1.7	6.6%
Caffeic Acid (nM)	550	1030	830	280	50.9%	530	820	530	0	0.0%
Ferulic Acid (nM)	4080	4001.4	10768.4	6688.4	163.9%	5587.6	14115.8	9316.1	3728.5	66.7%
Cyanidin-3-Glucoside (nM)	0.076	0.18 ^b	0.16	0.084	110.5%	0.14	0.35 ^b	0.26	0.12	85.7%
Protocatechuic Acid (nM)	2063.3	1570.2	1459.9	-603.4 ^a	-29.2% ^a	2264.5	1758.4	1408	-856.5 ^a	-37.8% ^a
Carotenoids (nM)	Not Detected	Not Detected	Not Detected	N/A	N/A	Not Detected	Not Detected	Not Detected	N/A	N/A

Discussion

The current study had found a statistically significant reduction in systolic blood pressure (-5.15%), diastolic blood pressure (-7.43%), and serum triglycerides levels (-14.34%) in study participants who had consumed 200g of purple carrots as part of their typical diet for a 12 week period when compared to the participants pre-intervention measures. However, statistically significant changes in the other measured outcomes in the study were not observed. Furthermore, no statistically significant differences were seen between any of the participants' pre- and post-intervention measures in participants who had consumed 200g of orange carrots as part of their typical diet for a 12 week period.

The following are the detailed discussions on selected study parameters:

Blood Pressure

The current study had shown a statistically significant reduction in systolic and diastolic blood pressure in the Purple Carrot group intervention group. These results are in agreement with previous studies using rat models (21, 22), a hamster model (23), and human trials (24, 25, 26, 27, 28).

Poudyal et al. (21) had demonstrated that a purple carrot juice supplementation in a high fat/high carbohydrate diet rat model was able to moderate increases in blood pressure and lower left ventricle fibrosis compared to those who did not receive purple carrot juice supplementation. This study also found that the supplemented rats had significantly less diastolic stiffness in comparison to the control group.

Similar results have also been shown in human trials following pomegranate juice and hibiscus sabdariffa tea consumption. In Aviram et al. (26), ten patients with carotid artery stenosis were supplemented with pomegranate juice for one year and 5 patients for up to 3 years and were compared to a control group that did not consume pomegranate juice. Systolic blood pressure was decreased by 21% following the first year of pomegranate juice consumption however failed to have an effect on diastolic blood pressure. In another study by Aviram et al. (27), daily consumption of 50ml anthocyanin-rich pomegranate juice for 2 weeks resulted in a 5% reduction in systolic blood pressure in hypertensive participants. Diastolic and systolic blood pressure reductions were observed in studies involving interventions with hibiscus sabdariffa (HS) tea. HS experiment groups had significantly greater reductions in systolic and diastolic blood pressure compared to placebo groups, black tea groups (28, 29, 30) and commonly used blood pressure medication, Captopril (31).

Although the mode of action for the observed effect on blood pressure cannot be confirmed in the current study, previous in vitro and in vivo studies may provide some insight of several potential mechanisms. One possible mechanism is anthocyanin's potential as a vasorelaxant (10). In a study examining the association vasodilation capacity, phenolic content and antioxidant activity of various red wines, anthocyanins were reported to be the only phenol to be linked with vasodilation capacity (24). This was further confirmed by Andriambeloson et al. (25) where anthocyanin containing fractions of red wine demonstrated vasodilation whereas phenolic acid derivatives and flavanol classes did not bring about the same response.

Furthermore, anthocyanins may also prevent vasoconstriction which can lead to a reduced blood pressure. Anthocyanin's well reported capacity as a powerful antioxidant may prevent free-radical and nitrogen specie-induced endothelium contractions and re-establish proper endothelial function (26,

27, 32). A similar effect can possibly achieve though anthocyanin's apparent ability to inhibit angiotensin converting enzyme (ACE) (27). ACE is involved in the production of angiotensin II, a strong vasoconstrictor, from angiotensin I, thus inhibition of this enzyme contributes to vasorelaxation. In addition, increased serum ACE activity has been correlated with an increased susceptibility to lipid peroxidation (33), thus inhibition through anthocyanin attenuates reactive oxygen-induced endothelium contraction.

Triglycerides

The function of serum triglycerides as a risk factor for cardiovascular disease has historically been contentious but recent studies have established a relationship between elevated serum triglycerides and coronary atherosclerosis (34, 35). In the present study, participants who had consumed 200g/d of purple carrots, containing 700mg C3G for a 12 week period were associated with a statistically significant decrease in serum triglycerides. These results are in agreement with studies where apo-E deficient mouse models and hyperlipidemic rats were supplemented with bilberry (15, 36), in hyperlipidemic patients who consumed red grapefruit for 30 days (16), in human RCTs with hibiscus sabdarriffa L. tea consumption (37), in rats fed purified Haskap fruit (38), following 30 days of strawberry consumption in humans (1), in humans following a short-term supplementation with whortleberry fruit (39), in KK-Ay mouse model supplemented with cyanidin-3-O- β -glucoside (40) and in pre-and post-menopausal women following a 4 week grape polyphenol intervention (41).

Animal studies involving mouse and rat models have demonstrated results similar to those found in the current study. Mauray et al. (15) had found that bilberry extract, an anthocyanin-rich fruit, had reduced serum and liver triglyceride levels in apo-E deficient mice. (40) had supplemented KK-Ay

mice, a Type 2 diabetes model in which under a standard chow diet would cause the mice to spontaneously become obese with fatty liver and hypertriglyceridemia, with cyanidin-3-O- β -glucoside. The cyanidin-3-O- β -glucoside rich diet had lowered serum triglyceride levels by 41.7% when compared to the control group. Rat models studies had reported similar decreases in serum triglyceride concentrations (38, 42).

A potential mechanism by which anthocyanin lowers serum triglycerides appears to involve the adenosine monophosphate-activated protein kinase phosphorylation (pAMPK)-lipoprotein lipase (LPL) signaling pathway, important in the regulation of triglycerides breakdown and thus in turn is important in the removal of serum triglycerides and fat metabolism in skeletal muscle and adipocytes. This hypothesis was investigated confirmed by Wei et al.(40); using a KK-Ay mouse model and skeletal muscle cells and adipocytes *in vitro*. It was suggested that cyanidin-3-O- β -glucoside (C3G), a common anthocyanin, had affected the activity of mRNA expression and protein expression of lipoprotein lipase (LPL). C3G supplementation had led to significant increases compared to the control mouse group in plasma and skeletal muscle LPL activity (35% and 26%, respectively) but a decrease in the visceral adipose tissue (37.9%). This result was supported *in vitro* which demonstrated similar results in LPL mRNA and protein expression and significant increases in pAMPK expression in skeletal muscle (2.2 fold) and visceral adipose tissue (2 fold). Therefore, based on this finding it can be suggested that anthocyanin consumption decreases circulating triglycerides by increasing LPL activity, through the activation of pAMPK, in the blood and skeletal muscle, thus increasing the breakdown of triglycerides in the blood and their subsequent metabolic oxidation in the skeletal muscle.

Furthermore, Seymour et al. (43) have suggested another mode of action through the activation of peroxisome proliferation-activated receptors (PPAR). PPAR is a transcription factor which targets

genes involved in fatty acid oxidation, tissue lipolysis and fatty acid transport, principally expressed in the liver and skeletal muscles. PPAR agonists, such as fibrates, can also lower blood lipids and change fat metabolism in adipose tissues. Using a Dahl-Salt Sensitive rat model, who become insulin resistant and hyperlipidemic without becoming obese, the study found that 90 day supplementation of anthocyanin-rich tart cherry, plasma triglyceride concentrations were lowered. Tart cherry supplementation had also significantly increased PPAR- α transcription level and PPAR- α target acyl-coenzyme A oxidase mRNA and activity. As tart cherry had similar effect on PPAR- α transcription levels as pharmacological PPAR- α agonist and had increased the expression and activity of acyl-coenzyme A oxidase, anthocyanins may reduce plasma triglyceride levels through PPAR- α /acyl-coenzyme A oxidase associated pathways.

Absorption, Distribution, Metabolism, Excretion

Data from unpublished manuscripts (Hala Ayoub from Dr. Meckling lab, University of Guelph) had reported on the phenolic content of the carrots used in the current study had found that the purple carrots contained significantly higher total phenolic, flavonoid, carotenoid, and anthocyanin content than the orange carrots. However, there was not a statistically significant difference in cyanidin 3-glucoside plasma content between the purple and orange carrot group. There could be two possible explanations for this observation. Firstly, blood samples were taken following 12-hour fast. As reported by Mazza et al. (44), anthocyanin glycosides and their derivatives are prominent in the plasma shortly after consumption (0-5 hours), after which they become methylated or undergo other metabolic transformations. As a result, the measures in the current study would not be able to detect the true concentration of anythocyanins that reached circulation since blood samples were draw after a 12 hour fast. This could be a possible explanation of why cyanidin-3-glucoside plasma concentrations were not significantly increased in the purple carrot group. Secondly, the small number of participants enrolled in

the study may have played a factor. With only 18 participants enrolled, the power analysis conducted prior to the start of the study required 30 participants to achieve 80% power to detect a 10% change in the measured biomarkers was not accomplished.

Although cyanidin 3-glucoside plasma concentrations had increased by an average of 87.5% in 9 participants following 12 weeks of purple carrot consumption, this was not a statistically significant result because of small sample size.

Cholesterol

The current study was not able to demonstrate the changes in LDL and HDL cholesterol that a number of previous human trials had found following a supplementation of anthocyanin extract or anthocyanin-rich fruits or vegetables. A study involving healthy volunteers had found that subjects who had consumed 500g/day of strawberries for 30 days had a significant reduction in LDL cholesterol (-13.72%), as well as triglyceride levels (1). In the same study, the researchers had determined that LDL cholesterol and triglyceride levels had returned to the pre-intervention measures following a 15 day washout period.

Qin et al. (14) had also found a decrease in LDL measures (13.6%) following 320 mg/day of purified anthocyanin supplementation for 12 weeks in dyslipidemic subjects. This study had also reported a 12.7% increase in HDL cholesterol levels in the anthocyanin group. Similarly, (45) had reported a significant 10% decrease in LDL cholesterol and 12.8% increase in HDL cholesterol in hypercholesterolemic participants following a 320 mg/day supplementation of purified anthocyanins for 12 weeks.

A potential explanation for the differing results in the current study may have to do with the nature of the supplementation and study participants. The current study's method of anthocyanin supplementation was carrots whereas Qin et al. (14) and Mauray et al. (45) had used capsules of purified anthocyanins from bilberry and black currant. As reported by Charron et al. (46), the amount of time for digestive processes to unfetter anthocyanins from the plant matrix of carrots may slow the rate of anthocyanin absorption when compared to purple juice. This differing rate of absorption and other possible roles of the plant matrix in affecting anthocyanin absorption could be attributed to the differing results between the two studies. In addition, the conditions of the participants in each study may have played a role in the differing results. In the current study, only 2 of the 9 participants in the purple carrot group were dyslipidemic, while all 60 participants in the anthocyanin group in Qin et al. (14) were dyslipidemic and all 75 participants in Zhu et al. (45) were hypercholesterolemic. As the majority of participants in the current study were considered to be the normal range for LDL and HDL cholesterol, being able to detect significant changes after the 12 week intervention would be less likely than being able to detect significant change those who were dyslipidemic and hypercholesterolemic prior to supplementation.

Inflammation

The current study did not find a significant change in inflammation biomarkers such as CRP, IL-6 and TNF- α following consumption of anthocyanin-rich purple carrots. This is in contrast with a few human studies which had found an inverse association between the consumption of flavonoids, such as anthocyanins, and inflammatory mediators. Using the 24 hour dietary recall and serum CPR data from the National Health and Nutritional Examination Survey, Chun et al. (47) had concluded that flavonoids intake was inversely related with serum CRP concentrations. Furthermore, numerous studies had

reported that plasma CRP, IL-6, and other pro-inflammatory chemokines, cytokines and inflammatory mediator concentrations were reduced following 3-6 week consumption of anthocyanins and anthocyanin-rich purple potatoes (13, 48, 49). It may be possible that the current study was unable to repeat the reported anti-inflammatory effects of anthocyanins because the sample size was not large enough to detect a significant change as the mentioned studies above had 22 to 60 participants in the anthocyanin groups. The current study had reported a 10.12% reduction in IL-6 plasma concentrations, but was not statistically significant. It is possible that 9 participants in the purple carrot group were too few to detect a statistically significant change.

Future Directions and Final Conclusions

In future studies, it may be advisable to narrow the inclusion criteria and target more specific cardiovascular disease risk factors. Based on the findings of the current study, it is suggested that a study should be designed to include only participants who have shown elevated blood triglycerides or separately with only hypertensive patients in order to fully understand the true extent that habitual consumption of purple carrots has on these measures. Furthermore, it can also be suggested that future studies should include a comparison between a study group consuming raw purple carrots and a study group consuming an equivalent amount of carrots in a juice form; it is postulated that this approach will also help to understand the role of rapid absorption as compared to slow absorption following digestion. Research done by Charron et al. (46) had found that the food matrix affects anthocyanin absorption.

The bioavailability of anthocyanins after consumption of purple carrots must be assessed in a different manner than the current study. Rather than drawing blood samples for the purpose of assessing bioavailability following a 12 hour fast, samples should be taken in two intervals such as within

5 hours after carrot consumption and at 12 hours. This proposal will be able to determine the blood level of anthocyanin at 5 hours and its true level at 12 hours post consumption, respectively. It will also determine if the anthocyanin level remains steady up to 12 hours or if it declines by subsequent tissue utilization, metabolism and excretion.

A gap in knowledge currently exists in regards to how anthocyanins are absorbed into the systemic circulation in humans. The current literature indicates that majority of anthocyanin absorption occurs at the stomach (50, 51, 52, 53) and small intestines (54, 55). Although nutrient absorption via the stomach is unusual, it has been established in rat studies where an *in situ* gastric administration of anthocyanin glycosylates were detected in portal and systemic circulation (50, 51), however, currently there is no direct evidence of this phenomenon taking place in human subjects and hence are need of further study.

In conclusion, the current study findings suggest that habitual consumption of anthocyanin-rich purple carrots may improve serum triglyceride levels, and systolic and diastolic blood pressure in individuals at risk for cardiovascular disease. Larger studies with only hypertensive or hypertriglyceridemic participants need to be conducted to better establish these observed effects. The effect of anthocyanin-rich foods to improve cholesterol levels and inflammation which was reported in previous animal and human studies was not able to be corroborated with the current study.

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