## ABSTRACT

OVERALL, JOHN CLAY. Anthocyanin Structural Diversity and Gut Microbiota Interactions Contribute to Divergent Metabolic Health Outcomes after Berry Nutritional Interventions in Diet-Induced Obesity (Under the Direction of Dr. Jonathan Allen).

The 2020-2030 Strategic Plan for NIH Nutrition Research emphasizes that an emerging priority for clinical and biomedical research efforts concerns the study of medical nutrition therapies, or the use of "food as medicine", for improved health outcomes and reduced clinical burden in chronic diseases. Nutritional modification/ intervention strategies may benefit specific patient populations with clinical risk factors for chronic disease by incorporating "medical foods" into the habitual diet; these foods, frequently plant in origin, are typically abundant sources of biologically active (bioactive) phytochemical compounds, and their intake as regular dietary components may possess therapeutic value with regard to the prevention and management of risk factors for cardiometabolic disease (CMD), obesity and type 2 diabetes (T2DM). Behavioral/lifestyle modification approaches based on dietary prescription are increasingly considered as viable strategies for risk factor management in preventable chronic disease and provide a valuable alternative therapeutic approach to conventional pharmacological intervention. Presently, it is critical that research efforts address the identification, development, and optimization of new and effective candidates for medical nutrition interventions. Particularly promising in this respect are fruits and vegetables constituting significant sources of therapeutic phytochemical compounds, especially the (poly)phenolic flavonoid class and more specifically, the anthocyanins (ANC), a major flavonoid subclass that is abundant in commonly consumed colorful fruits and vegetables, and most especially in various species of berry fruits.

CMD risk factors are important therapeutic targets for dietary interventions with ANC-rich berries, but gaps remain in our current understanding. The molecular and physiological

mechanisms responsible for their effects on cardiometabolic endpoints are not well understood, and we lack proper understanding of whether specific ANCs differ significantly in bioactivity relative to other ANC structures, and of the extent of these differences should they indeed be of physiological significance. Furthermore, dietary intake of ANC-rich berries has not proven therapeutic efficacy with respect to cardiometabolic endpoints across all studies, complicated by literature hampered by incomplete understanding of their bioavailability and lack of consideration for their metabolic fate after ingestion in humans, as well as by the complexities added by the format in which they are consumed (i.e. as whole berries or purified dietary supplements).

This thesis aims to delineate existing scientific understanding about this complex subject and provides experimental data that builds on the current gaps in knowledge through examining impacts of dietary supplementation with six different berries containing structurally diverse ANC profiles in a mouse diet-induced obesity (DIO) model driven by high-fat diet (HFD), an obesity model suitably translatable to human obesity-related CMD. Blackberry (mono-glycosylated cyanidins), black raspberry (acylated mono-glycosylated cyanidins), blackcurrant (mono- and di-glycosylated cyanidins and delphinidins), maqui berry (di-glycosylated delphinidins), Concord grape (acylated mono-glycosylated delphinidins and petunidins), and blueberry (mono-glycosylated delphinidins, malvidins, and petunidins) were normalized to 400 μg/g total ANC content for dietary supplementation and differentially affected development of metabolic risk factors in the C57BL/6 mouse model of polygenic obesity; their bioactive discrepancy in this model could be explained by differences in their key representative ANC structures affecting their absorption and metabolism by host and gut microbes. Consumption of berries also resulted in a strong shift in the gastrointestinal bacterial communities towards obligate anaerobes that

correlated with decrease in the gastrointestinal luminal oxygen and oxidative stress. Further work is needed to elucidate the relative contributions of host, diet and/or microbial oxidative activity to biological effects of structurally diverse berries and the implication of these differences to human cardiometabolic health.

# Anthocyanin Structural Diversity and Gut Microbiota Interactions Contribute to Divergent Metabolic Health Outcomes after Berry Nutritional Interventions in Diet-Induced Obesity

by
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## BIOGRAPHY

John Clay Overall was born in Washington, D.C., on September 24, 1993 to Scott Clay and Xiumin Huang Overall. Following his older brother Jesse, he graduated from the Bullis School in Potomac, MD in 2011. He majored in Biology at Davidson College and received a B.S. in 2015 and subsequently began to pursue graduate study in nutrition at North Carolina State University. John's foray into biomedical research began in Fall 2015 when he started as an intern research assistant at the Plants for Human Health Institute (PHHI) in Kannapolis, NC. He joined the Nutrition Graduate Program of the NCSU Department of Food, Bioprocessing, and Nutrition Sciences (FBNS) in 2016. Following completion of his Master's degree, John will explore opportunities in public health, healthcare, food, agriculture, or the pharmaceutical industry. A long-time health enthusiast, John enjoys learning, experimenting with, and circulating old as well new concepts in health promotion and healthy aging, nutrition, fitness, integrative health, traditional medicine. John enjoys giving advice and complaining how the days are too short.

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For my parents, to whom I owe everything.

"*It is not always in this world the people who bring us fine roses to whom we are most friendly.*"

- Proust

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# CHAPTER 1. REVIEW OF RELEVANT LITERATURE

## 1.1. Introduction

High-fat diet (HFD) is a key factor in the pathogenesis and progression of cardiovascular and metabolic disorders – also known as cardiometabolic disease (CMD). CMD is tightly linked with HFD-associated obesity, insulin resistance and type 2 diabetes (T2DM), and chronic metabolic inflammation (Wali *et al.* 2020). Cardiometabolic risk factors are closely associated with the metabolic syndrome (MetS) and are linked by a common pathophysiology that involves adipose tissue dysregulation and ongoing inflammatory burden (sterile or chronic inflammation) (Aguilar-Salinas & Viveros-Ruiz 2019). Chronic inflammation is characterized by sustained low-level activation of the acute pro-inflammatory response which skews the systemic balance towards a proinflammatory state through undue activation of immune cells, proinflammatory (M1) macrophage phenotype switching, and excessive cytokine secretion (Appari, Channon, & McNeill 2018). Chronic inflammation is associated with many of the etiological drivers that are compounded by dysregulated lipid metabolism and ectopic lipid deposition in organs like skeletal muscle (Chen *et al.* 2019; Small *et al.* 2018), and generally accompanying shifts in cellular substrate use and energy metabolism (bioenergetic function) that are associated with mitochondrial functional impairment (Gao *et al.* 2020a). Ultimately, obesity-related CMD development occurs through a dynamic process in which complex and fluctuating contributions from genetic, behavioral, and environmental factors drive in tandem the progressive structural and functional deterioration of metabolically active tissues and organ systems - such as in liver, adipose tissues, and skeletal muscle –that leads eventually to loss of normal function of these key tissues and organ systems, with progressively detrimental consequences for host physiology

(Mechanick *et al.* 2020). Left unchecked, these etiological determinants of CMD lead to development of clinical risk factors and progression of CMD-associated morbidity.

Diets rich in plant foods, especially fruits and vegetables, are associated with fewer and less severe adverse cardiometabolic health outcomes in comparison with diets that are less plant-based. The unique health benefits of fruits and vegetables can be attributed to the hugely diverse array of phytochemicals that they exhibit. One class that has been particularly well studied owing to its ubiquity in the plant kingdom is the phenolic compounds, a group of plant secondary metabolites that exhibit diverse structural characteristics and biological activities. Polyphenols, especially of the flavonoid subclass, are found in the human diet in abundance and have been associated with a broad range of health effects. Research on the flavonoids has generated particular interest in the anthocyanin (ANC) subclass. ANCs are a group of water- soluble pigments responsible for the red, blue, and purple colors exhibited by a variety of fruits and vegetables. Over 700 structurally distinct ANCs have been identified, with most (~90%) occurring as glycosides of six common anthocyanidins: pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt), and malvidin (Mv) (Jordheim, 2010). This chapter presents recent evidence to demonstrate that different ANC aglycones can differ markedly in terms of bioavailability, absorption, metabolism, metabolic breakdown products, and excretion to produce wide-ranging impacts on human metabolic health and disease.

Several epidemiological studies have reported that the intake of ANCs is associated with antidiabetic, anti-inflammatory, and cardioprotective effects (Cassidy et al., 2011; Wedick *et al.*, 2012; Cassidy *et al.*, 2013, 2015). Estimates of the daily average anthocyanin consumption in populations of US and Spanish adults range from 12.5 to 18.8 mg (Wu *et al.*, 2006; Zamora-Ros *et al.*, 2010). Fruits and vegetables are reported to contain total ANCs at levels ranging from 0.7

to 1734 mg per 100 g fresh weight (FW). The incorporation of a single serving of ANC-rich fruit (e.g., berries) in the diet can contribute up to hundreds of milligrams of ANCs; as a result, substantial increases in ANC intake are readily achievable through relatively small dietary modifications.

In light of the therapeutic potential of ANCs and their relative abundance in the human diet, many in vitro and in vivo studies have been conducted with the aim of elucidating not only the biological effects of ANCs, but also their disposition and metabolism after ingestion. The biological effects of ANCs seem to depend highly upon structure, as marked differences have been observed among the common ANC aglycones. The relative potency of different ANCs in causing beneficial effects on energy and substrate metabolism is therefore evaluated in the following sections.

**1.2. Chemical structure**

ANCs are polyhydroxylated or polymethoxylated glycosides of anthocyanidins (aglycones), which are oxygenated derivatives of flavylium (2-phenylbenzopyrylium) salts (Jordheim, 2010). The basic structure of anthocyanidins is represented by an aromatic ring (A) bound to a heterocyclic ring containing oxygen (C), which bears in turn a third aromatic ring (B). ANCs are connected with sugar moieties via O-linkages at the 3-position; additional moieties may link to the anthocyanidin through their 5-, 7-, 3′-, 4′-, or 5′-hydroxyl substituents. The sugar moieties are mono-, di-, or trisaccharides comprising one or more glucosyl, galactosyl, rhamnosyl, arabinosyl, xylosyl, glucuronosyl, and apiosyl units; glucosyl units are the most ubiquitous and at least one glucosyl unit is present in 90% of the various ANCs. The sugar moiety, which increases the stability of the anthocyanidin and its solubility in water, may be further acylated with aromatic and/or aliphatic acyl groups. These acylations typically occur at

the 6-position of the sugar moiety (86%), but have also been reported at the 2-, 3-, and 4-positions. In general, fruits primarily contain simple ANCs (with only one or two monosaccharide units and no acylations), whereas in vegetables, complex ANCs are much more likely to predominate (Jordheim, 2010). As discussed later in this review, the complex glycosylation and acylation patterns exhibited by anthocyanin structures in vegetables, relative to fruits, tend to associate with increased gastrointestinal stability, lower bioavailability, and possibly, lower bioactivity (Charron *et al.*, 2009). Most fruit and vegetable species tend to contain ANCs based on only one primary anthocyanidin, usually Cy; notably, strawberry contains primarily Pg-based ANCs. Some species may contain ANCs based on two anthocyanidins, such as blackcurrants, which contain similar distributions of Dp- and Cy-based ANCs; still other species contain a range of anthocyanidins, such as blueberries and bilberries, in which ANCs are found based on all the common anthocyanidins except for Pg. The common ANC structures differ in their patterns of hydroxyl and methoxy substitution on the B-ring at the 3′- and 5′-positions. Mv is the most methoxylated (3′-OCH3 , 5′-OCH3 ), followed by Pn (3′-OCH3 , 5′-H), and Pt (3′-OH, 5′-OCH3 ), which also bears a hydroxyl group; Cy (3′-OH, 5′-H) is similarly monohydroxylated. Pg (3′-H, 5′-H) is unique in that it is neither hydroxylated nor methoxylated (Figure 1.1).

In aqueous solution, anthocyanidins exist in pH-dependent equilibrium as four molecules: the red flavylium cation (pH <4), colorless hemiacetal/carbinol pseudobase (pH = 4–5), violet–blue quinoidal base (pH = 6–8), and yellow chalcone (pH 8). Increasing pH leads to competition between deprotonation, forming the blue quinoidal structure and the hydration of the flavylium cation, giving the colorless hemiacetal form at pH >4 (Figure 1.2). The hemiacetal form undergoes C-ring opening by a tautomeric process to form the yellow cis-chalcone, which

ultimately isomerizes to the trans-chalcone, which degrades to form phenolic acids (Jordheim 2010).

### 1.3. Structural effects on stability

ANC stability is strongly affected by external factors such as oxygen, temperature, light, enzymes, and pH (Jordheim 2010). The structure of ANCs determines their susceptibility to degradation in vivo and thus influences the disposition and metabolism of ANCs. The distribution of the various secondary structures under in vivo conditions is likely to vary with both the specific physiological conditions and the structure of the ANC. Under the acidic conditions of the stomach, ANCs are likely present as flavylium cations, whereas the other forms (hemiacetals, chalcones, quinones) are likely to predominate at the more alkaline pH lower down the gastrointestinal (GI) tract and in the blood. These forms are considerably less stable and likely mediate the spontaneous chemical degradation of ANCs to phenolic acids. In vivo, anthocyanin deglycosylation occurs rapidly and extensively to produce anthocyanidins that have significantly less molecular polarity as well as enhanced membrane solubility, enabling paracellular uptake of anthocyanidins into cells in a transport process that occurs independently of membrane transporters (Kalt, 2019).

Glycosylation broadly affects the stability and absorption efficiency of the corresponding ANCs, and this has been well established in animal models. Galactosides from bilberry ANCs remained in rat plasma for longer than glucosides (Ichiyanagi *et al.*, 2006), and Cy- glucosides were significantly more absorbed in rat intestine compared with Cy-galactosides and -rutinosides (Talavera *et al.*, 2004). In pigs, rutinosides and sambubiosides of ANCs from marionberry, elderberry, or blackcurrant were primarily (>80%) excreted intact (Wu *et al.*, 2004, 2005). Acylated ANCs are also significantly less bioavailable than non-acylated ANCs, as shown in a

study of purple carrot juice in human volunteers where non-acylated ANCs achieved peak plasma levels that were fourfold higher compared with acylated ANCs, despite the fact that acylated ANCs comprised 76% of the total ANCs in the juice (Charron *et al.*, 2009). Another study of purple carrot similarly found that non-acylated ANCs were absorbed more efficiently (0.12–25%) than acylated ANCs (0.0079–0.019%) (Novotny *et al.*, 2012). Furthermore, only intact acetylated ANCs, and not their respective conjugated metabolites, were detected in blood and urine; taken together, the results indicate that the absorption of complex ANCs is limited compared with simple ANCs.

The aglycone structure modulates the stability of ANCs under physiological conditions. An in vitro investigation of the degradation of purified ANCs in simulated intestinal fluid showed that Pg-3-glucoside exhibited the slowest degradation and Dp-3-glucoside exhibited the fastest, indicating that increased B-ring hydroxylation decreases ANC stability (Woodward *et al.*, 2009). Fleschhut *et al.* (2006) evaluated the degradation of Mv, Cy, Pg, Dp, and Pn aglycones in neutral (pH=7.4) media, observing the near total disappearance of all ANCs after 60 min with the exception of Pg, of which only 20% was degraded. It was speculated that aglycone stability under neutral pH conditions is negatively correlated with increasing hydroxy or methoxy substitutions on the B-ring. Therefore, it was proposed that Pg might be the most stable anthocyanidin.

Ichiyanagi *et al.* (2006) reported that the time to half plasma clearance of bilberry ANCs in rats followed the order Dp>Cy>Pt=Pn>Mv. The major metabolites observed after bilberry ANC ingestion were O-methyl-ANCs (Pt, Pn, and Mv glycosides) and the O-methylated metabolites of the parent ANCs (Dp- and Cy glycosides). These results suggested that O-methylation lends hydrophobicity to the B-ring of ANCs, thus increasing the tissue affinity of

ANCs and reducing the plasma residence time. As a result, ANCs with free OH groups in the B-ring (Dp and Cy glycosides) must theoretically be converted into O-methyl metabolites before they are distributed into tissues (Ichiyanagi *et al.*, 2006). More recently, Stalmach *et al.* (2012) found that glycosides of Mv (57%), Pn (48%), and Cy (37%) were significantly more stable during in vitro gastric and pancreatic digestion than were glycosides of Pt (14%) and Dp (5.5%). There was increased recovery of the O-methyl-ANCs (Pt, Pn, and Mv glycosides) in ileal fluid compared with conjugates of Dp or Cy, further supporting the stabilizing effects conferred on ANCs by the O-methyl structure.

It has been proposed that the presence of ortho-positioned hydroxyl groups on the B-ring is required for ANCs to undergo O-methylation by catechol-O-methyl transferase (COMT). Consequently, O-methylation may be specific to Cy-3-glc and Pt-3-glc, which have a catechol B-ring, in addition to Dp-3-glc, which has a pyrogallol ring. In rats, Cy-3-glc was metabolized to preferentially 3′- but also 4′-O-methyl-Cy-3-glc, whereas Dp-3-glc was metabolized to 4′-O-methyl Dp-3-glc (Ichiyanagi *et al.*, 2004, 2005). It has been reported that Pt-3-glc forms Mv-3-glc after O-methylation by COMT (Zimman and Waterhouse, 2002). Theoretically, Dp-based ANCs might convert in vivo to Mv-based ANCs through repeated O-methylation. Taken together, it appears that Cy and Pn glycosides, and Dp, Pt, and Mv glycosides, might undergo interconversion in vivo due to O-methylation. Pg-based ANCs do not undergo O-methylation and therefore are more extensively converted to metabolites retaining the aglycone structure (Felgines *et al.*, 2007; Carkeet *et al.*, 2008). The fact that Pg has only one hydroxyl group on the B-ring increases its availability for glucuronidation.

Talavera *et al.* (2004) reported that the presence of methoxy substituents on the B-ring reduced the intestinal absorption of bilberry ANCs in rats; Mv-3-glc was the least absorbed

(10.7%) ANC and Cy-3-glc was the most highly absorbed (22.4%). ANCs from blueberry, grape, and sour cherry have been observed to transport in their intact form across Caco-2 cell monolayers, albeit with relatively low efficiency (0.0005–4%) (Yi *et al.*, 2006; Faria *et al.*, 2009; Toydemir *et al.*, 2013; Kuntz *et al.*, 2015a,b). In contrast, blackcurrant ANCs, Dp-3- glc from grape extract, and diglucosides of Mv and Pn from grape/blueberry extract were not transported through Caco-2 monolayers (Steinert et al., 2008; Kuntz et al., 2015a,b). Therefore, it seems that anthocyanidin structure is a key determinant of transport efficiency across enterocytes, such that fewer hydroxyl groups and more methoxy groups improve stability during intestinal digestion and increase absorption efficiency (Liu *et al.*, 2014b). Consistent with this, several studies have found that Mv-3-glc is transported with high efficiency compared with other ANCs, whereas Dp-3-glc is absorbed either with the lowest relative efficiency or not at all (Yi *et al.*, 2006; Liu *et al.*, 2014b, Kuntz *et al.*, 2015b). This has been attributed to the greater hydrophobicity conferred by the methoxy substituents compared with hydroxyl substituents, as Mv is the most hydrophobic ANC and Dp is the least. Pg-3-glc has also been shown to cross Caco-2 cell monolayers, although only in trace amounts (Kosinska-Cagnazzo *et al.*, 2015).

In contrast, gastric absorption appears to be unaffected by aglycone structure; in human gastric epithelial MKN-28 cells, Dp-3-glc, Cy-3-glc, and Mv-3-glc were each significantly absorbed to a similar extent (Fernandes *et al.*, 2012). This is consistent with the high stability of ANCs under acidic conditions.

### 1.4. Systemic bioavailability and tissue distribution

Until very recently, most ADME (absorption, distribution, metabolism, and excretion) studies in humans and animals have indicated that ANCs are very poorly available in vivo. Based on the results of 97 ADME studies published before 2005, acute ANC ingestion in humans leads

to a Cmax of 0.03 μmol/l, which is reached on average at 1.5 h (= Tmax) post-ingestion; <0.1% of the ingested ANCs were typically recovered in urine (Manach *et al.*, 2005). McGhie *et al.* (2003) administered a mixture of boysenberry, blackcurrant, and blueberry to human participants and reported that urinary excretion of intact glycosides accounted for 0.01–0.06% of the ingested ANCs after 7 h. In addition to intact ANCs, ANC metabolites retaining the parent aglycone structure (conjugates of ANCs and/or aglycones) were observed in other studies (Wu *et al.*, 2002; Felgines *et al.*, 2003, 2005; Kay *et al.*, 2004). For example, Felgines *et al.* (2005) reported a total urinary excretion of 0.14–0.18% for blackberry ANC metabolites, which occurred as intact ANCs and aglycones in addition to their methylated, glucuronidated and/or sulfated conjugates. The low levels of ANCs present in plasma and urine relative to ingested ANCs led to the prevailing assumption that ANC are inefficiently absorbed (Manach *et al.*, 2005; McGhie and Walton, 2007). However, these studies offer limited interpretive value because they only measured recoveries of intact and conjugated ANCs.

In more recent years, stable isotope labeling has provided new insights into the complex issue of ANCs disposition after ingestion (Figure 1.3 and Figure 1.4). Most notably, Czank *et al.* (2013) established the relative bioavailability of Cy-3-glc as 12.4%, accounted for by the recoveries of 5.4% and 6.9% of the $^{13}C$ label in urine and breath, respectively. Hence the bioavailability of ANCs is likely to be considerably higher than was previously suggested. It is clear that ANCs are metabolized extensively following ingestion and therefore are present in the circulation in substantially higher concentrations compared with parent ANCs, indicating that the biological activity of ANCs may be mediated in large part by their metabolites. Although an ongoing area of research that is beyond the scope of the present review, Chandra *et al.*, (2019) presents a comprehensive illustration of the current prevailing theory regarding the bioactivity of

ANC metabolites versus parent glycosides. The poor apparent bioavailability of ANCs observed in previous studies arose because the authors did not account for the extensive degradation of ANCs in vivo to low molecular weight phenolic metabolites (Kay *et al.*, 2009). ANC degradation might occur spontaneously owing to the instability of ANCs at physiological pH, as initially proposed by Woodward *et al.* (2009). Alternatively, it has been proposed that the degradation of some ANCs is mediated largely by gut microflora, implicating the colon as the major source of ANC metabolites following ingestion (McGhie and Walton, 2007; Williamson and Clifford, 2010). In both cases, the extent of degradation and the type of metabolites produced are highly influenced by the ANC structure. Extensive presystemic metabolism in the intestine wall or liver has also been proposed to explain the low apparent bioavailability of ANCs such as Cy-3-glc and Pg-3-glc (Fang, 2014). A diagram illustrating the digestion, absorption, uptake and distribution of ANCs after oral ingestion is presented in Figure 1.5.

ANC deposition in various tissues has been studied in animal models; the mechanism of uptake is unknown although it is theorized that deglycosylation into less polar anthocyanidins is necessary for in vivo cellular uptake to occur, as a means of bypassing carrier-mediated transport (Kalt, 2019). Several studies have demonstrated that detectable levels of ANCs occur in brain tissue after ingestion; the neuroprotective effects of ANCs and their metabolites are beyond the scope of this review but are reviewed in Henriques *et al.* (2020). Andres-Lacueva et al. (2005) first detected Cy-3-gal, Cy-3-ara, Mv-3-gal, Mv-3-glc, Mv-3-ara, Pn-3-ara, and Dp-3-gal in the brains of rats administered a diet supplemented with blueberry extract for 10 weeks. Rat brain tissue contained 0.21 nmol/g Cy-3-glc after 15 days of blackberry feeding (Talavera *et al.*, 2005). In pigs fed a 4% blueberry diet for 4 weeks, 0.878 pmol/g total ANCs were found in the cortex and 0.664 pmol/g in the cerebellum (Kalt *et al.*, 2008). Following up on the earlier study,

Milbury and Kalt (2010) fed pigs 2% freeze-dried blueberry powder for 8 weeks and ultimately detected 0.70, 0.87, and 0.92 pmol/g of ANCs and glucuronides combined in the frontal cortex, cerebellum, and midbrain respectively. Recently, bilberry ANC metabolites were detected in the brains of weanling piglets following 3 weeks of bilberry supplementation at concentrations ranging from 0 to 3 pmol/g (Chen *et al.*, 2015). Interestingly, all ANC glycosides except Dp were shown to increase dose dependently in the cerebellum, despite the fact that Dp glycosides were the most abundant ANCs in the initial extract. Sakakibara et al. (2009) failed to detect ANCs in the brain, despite their significant presence in other tissues.

In addition to the brain, ANCs are consistently detected in significant amounts in the kidneys and/or liver; the latter has been suggested to be the primary target for ANC accumulation. One study estimated that 51.5% of ingested ANCs were contained in the liver (Sakakibara *et al.*, 2009). It is well established that ANCs reach far greater concentrations in GI tissues than in plasma (Talavera *et al.*, 2005; Marczylo *et al.*, 2009). Esposito *et al.* (2015) recently observed ANC concentrations of up to 195 μg/g in the jejunal tissue of C57BL/6 J mice after chronic intake of blackcurrant extract. ANCs have been detected in testicular and prostate/bladder tissue after ingestion in rodents and in ocular tissue in pigs (Kalt *et al.*, 2008; Felgines *et al.*, 2009; Marczylo *et al.*, 2009). Felgines *et al.* (2009) also identified blackberry ANCs in the heart and in adipose tissue, and Sakakibara *et al.* (2009) detected a low concentration (116 pmol/g) of ANCs in mouse lung.

*1.4.1. Absorption from the stomach and small intestine*

It was established early on that ANCs are absorbed and excreted in their intact glycosidic form after oral administration in humans (Cao and Prior, 1999; Miyazawa *et al.*, 1999; Cao *et al.*, 2001; McGhie *et al.*, 2003; Matsumoto *et al.*, 2006). Anthocyanin administration via intubation

directly into the stomach or the jejunum of patients confirmed absorption under both conditions; notably, intubation into the stomach resulted in a fivefold greater urine concentration of ANCs, which could be explained by the stability of ANCs under acidic conditions (Cai et al., 2011). After blackberry ingestion in rats, Cy-3-glc was rapidly absorbed from the stomach and was subsequently detected in the plasma (Talavera *et al.*, 2003). In rats administered grape ANCs, Mv-3-glc was detected in the portal (0.65 μM) and systemic (0.23 μM) circulation after just 6 min; the organic anion carrier bilitranslocase was proposed to mediate the transport of ANCs across the gastric mucosa (Passamonti *et al.*, 2003).

ANCs are also significantly absorbed from the small intestine. Between 10.7 and 22.4% of ANCs were absorbed following in situ perfusion of the jejunum and ileum in rats, depending on the anthocyanidin structure (Talavera *et al.*, 2004). He *et al.* (2009) similarly reported that black raspberry ANCs were significantly taken up by the small intestine in rats, ultimately accounting for 7.5% of the initial dose. In contrast to stomach tissue, which was found to contain only intact ANC, jejunal tissue contained not only intact ANCs but also their corresponding aglycones and phase II metabolites (Talavera et al., 2005). Since ANCs were recovered solely as intact glycosides in rat intestinal lumen after perfusion (Talavera *et al.*, 2004), it is unlikely that ingested ANCs are deglycosylated by intestinal enzymes such as cytosolic β-glucosidase or lactase-phlorizin hydrolase, as is the case with other flavonoids such as quercetin-3-glucoside. The mechanism responsible for the deglycosylation of ANCs in the small intestine has yet to be identified.

Some ANCs may be absorbed in the small intestine through interactions with glucose transporters and therefore may modulate intestinal glucose absorption; an updated review on the mechanisms involved is presented in Solverson (2020). Prolonged exposure of an ANC-rich

berry extract (Cy, Dp, Pt, Mv) to Caco-2 cells inhibited both total (SGTL1+GLUT) and facilitated (GLUT only) glucose uptake and also significantly reduced glucose transporter expression (Alzaid *et al.*, 2013). Pharmacological inhibition of SGLT1 and GLUT2 significantly decreased the absorption of Cy-3-glc in Caco-2 cells (Zou *et al.*, 2014). Pg-3-glc from strawberry extract was also reported to be a potent inhibitor of SGLT1 and especially of GLUT2 at physiological concentrations achievable after dietary ingestion (Manzano and Williamson, 2010). These findings suggest that the absorption of some ANCs in the small intestine may be dependent upon interactions with SGLT1 and GLUT2.

Following intestinal absorption, aglycones undergo extensive metabolism by phase II enzymes in the intestine wall and/or liver, followed by excretion via kidneys. Phase II metabolism involves UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), and catechol- O-methyl transferase, which catalyze the conjugation of ANC metabolites with glucuronic acid, sulfate, and methyl groups, respectively (Del Rio *et al.*, 2013). There is evidence that some phase II metabolites from the liver may undergo enterohepatic recirculation due to bile secretion to the small intestine (Fang, 2014).

*1.4.2. Absorption from the colon*

It is possible that considerable amounts of ingested ANCs can reach the colon; in ileostomy patients fed raspberries or Concord grape juice, 5-40% of ingested ANCs were recovered in the ileal fluid, depending on ANC structure (Gonzalez-Barrio *et al.*, 2010; Stalmach *et al.*, 2012). Recent studies in healthy animals and human subjects have reported that a significant proportion of ingested ANCs reach the colon without modification, where they are extensively metabolized by gut microflora (Gonzalez-Barrio et al., 2011; Czank et al., 2013; de Ferrars *et al.*, 2014; Esposito *et al.*, 2015). In vitro studies have demonstrated that gut microflora

catalyzes the hydrolysis of ANCs through cleavage of 3-glycosidic linkages. The resulting aglycones undergo subsequent degradation by fission of the C-ring, and the resulting A- and B-ring fragments yield a range of phenolic acids and aldehydes (Aura *et al.,* 2005; Keppler and Humpf, 2005). The low molecular weight phenolic metabolites generated by gut microbial metabolism are then absorbed into the large intestine wall and undergo subsequent phase II metabolism locally or in the liver (Fleschhut *et al.,* 2006). However, based on the 32% fecal recovery of $^{13}$C-labeled Cy-3-glc reported by Czank *et al.* (2013), it has been suggested that a significant proportion of colonic ANCs may not be absorbed into the circulation and are excreted in the feces.

In pig cecum after 25 min to 2 h, Cy, Mv, and Pn aglycones were hydrolyzed to their respective major degradation products, namely protocatechuic acid (PCA), syringic acid (SA), and vanillic acid (VA) (Keppler and Humpf, 2005). Purified Mv-3-glc, Pn-3-glc, and Cy-3-glc yielded SA, VA, and PCA, respectively, after 2 h of incubation with human fecal microflora (Fleschhut *et al.,* 2006). In another study, incubation of an ANC extract from Cabernet Sauvignon grapes (containing Dp-3-glc, Pt-3-glc, Pn-3-glc, and Mv-3-glc) with pig large intestine yielded the microbial metabolites 3-O-methylgallic acid, SA, and 2,4,6-trihydroxybenzaldehyde (phloroglucinol aldehyde), a putative A-ring metabolite. Although gallic acid is presumed to be the major metabolite of Dp degradation, as evident from intestinal wall extracts (Esposito *et al.,* 2015), it was not detected in pig large intestine despite the initial Dp-3-glc concentration, indicating that gallic acid itself might be efficiently absorbed and/or undergo rapid further degradation (Forester and Waterhouse, 2008). A more recent study observed gallic acid, SA and p-coumaric acid as the major degradation products after incubation of a mixture of Mv-3-glc, Dp-3-glc, Pn-3-glc, Pt-3- glc, and Cy-3-glc with fecal bacteria; individual incubation

of Mv-3-glc led to the formation of SA as a major metabolite (Hidalgo *et al.*, 2012a). The major degradation product of Pg-3-glc was 4-hydroxybenzoic acid (Azzini *et al.*, 2010).

The susceptibility of ANCs to gut microbial metabolism seems to be highly affected by anthocyanidin structure (Esposito *et al.*, 2015). In order to investigate the gastrointestinal distribution of blackcurrant ANCs, blackcurrant extract was administered chronically to C57BL/6 J mice either with or without antibiotics to disrupt the gut microbiome. Antibiotic treatment resulted in 16–25-fold increases in fecal ANC content, with Cy-based ANCs increasing the most dramatically and Dp-based ANCs showing comparatively smaller increases. In another study of mice fed a bilberry-enriched diet, Jakesevic et al. (2013) observed that absorption of Dp-3-glc and Cy-3-glc was largely bacterially independent, mostly occurring in the stomach and jejunum. Esposito et al. (2015) reported that up to threefold higher concentrations of gallic acid were observed in the jejunum compared with PCA, an effect that appeared to be independent of gut microbiome status. Since similar concentrations of Cy- and Dp-based ANCs were present in the initial extract and diet; this seemed to indicate that gallic acid was preferentially absorbed over PCA. The selective absorption of gallic acid compared with PCA, in addition to the different sensitivities of Cys and Dps to gut microbial metabolism, might explain in part the different biological effects of Cy- and Dp-containing berries.

Other minor degradation products of ANCs in the colon include catechol, resorcinol, pyrogallol, tyrosol, 3-(3′-hydroxyphenyl)propionic acid, dihydrocaffeic acid, and 3-(4′-hydroxyphenyl)lactic acid. In addition, various phenolic acid metabolites associated with ANC intake include ferulic acid, 4′-hydroxymandelic acid, homovanillic acid, hippuric acid, and 4′-hydroxyhippuric acid (Gonzalez-Barrio *et al.*, 2011; Czank *et al.*, 2013; de Ferrars *et al.*, 2014; Rodriguez-Mateos *et al.*, 2014).

## 1.5. Metabolism and nutrigenomic effects

Modern nutritional supplementation with anthocyanins aimed at metabolic health promotion and disease risk reduction revealed several nutrigenomic biomarkers that might explain how an anthocyanin-rich diet influences gene transcription, protein expression, and metabolism. Taken together, these new findings provide a strong scientific basis for developing new anthocyanin-rich foods, cultivars of existing crops, and dietary supplements adapted to the specific needs of consumers and targeted to a specific group of metabolic risk factors.

### *1.5.1. Pelargonidin (strawberry as main source)*

Strawberry feeding studies in animals and humans indicated that Pg-3-glc is metabolized differently to most other ANCs. After feeding marionberry (containing Cy-3-glc, Cy-3-rut, and Pg-3-glc) to pigs, the total recovery of ANCs in the urine (as parental, glucuronidated, and methylated forms) was considerably higher for Pg-3-glc (0.583%) than for Cy-3-glc (0.087%) or Cy-3-rut (0.084%); moreover, the majority of ingested Cy-3-glc and Pg-3-glc was excreted in the urine as metabolites (Wu *et al.*, 2004). Felgines *et al.* (2003) fed strawberries to human volunteers and recovered 1.8% of Pg-3-glc from the urine, primarily as one (80%) of several pelargonidin glucuronides, in addition to smaller amounts of a pelargonidin sulfate, intact Pg-3-glc, and aglycone Pg. Other studies recovered 0.75–2% of strawberry ANCs in the urine of volunteers after strawberry feeding; Pg-glucuronide was the major metabolite in both studies (Carkeet *et al.*, 2008; Mullen *et al.*, 2008). In a recent study of strawberry-fed rats, Pg-3-glucuronide and Pg-3-glc-glucuronide were identified as the dominant metabolites (Ichiyanagi *et al.*, 2013). It has also been proposed that Pg-3-glc undergoes phenolic degradation. Approximately 54–56% of ingested Pg-3-glc was found as its phenolic metabolite 4-

hydroxybenzoic acid (4HB) following strawberry feeding in healthy human volunteers (Azzini *et al.*, 2010).

Spontaneous degradation under neutral physiological conditions appears less relevant for Pg-3-glc than for other ANCs, as Pg-3-glc was shown to be relatively stable under simulated intestinal conditions (Woodward *et al.*, 2009). However, it was shown in vitro that human liver microsomes efficiently metabolize Pg-3-glc to 4HB, and then further metabolize 4HB into two glucuronide conjugates (Woodward et al., 2011). Taken together, the evidence suggests that Pg-3-glc undergoes significant phase II metabolism to a greater extent than other ANCs; however, phenolic degradation appears to be the primary metabolic pathway of Pg-3-glc in vivo.

In a series of experiments, Prior and co-workers found that the inclusion of purified strawberry ANCs in the drinking water of C57BL/6 J mice fed high fat diet (HFD) prevented the development of obesity and dyslipidemia, whereas supplementation with whole powdered strawberries did not and had only marginal effects on plasma glucose levels (Prior *et al.*, 2008, 2009). Supplementation with dietary strawberry powder (human dose equivalent of one strawberry serving per day) for 24 weeks significantly lowered blood glucose concentrations in both lean and diet-induced obese C57BL/6J mice, in addition to attenuating plasma C-reactive protein (CRP) concentrations in the lean mice (Parelman *et al.*, 2012).

A number of studies have suggested the weak potential for strawberries to modulate inflammation, glycemic response, and lipid status in human subjects, after either acute or chronic intake. In a crossover study of 20 obese human subjects, 3-week dietary intervention with strawberry powder reduced cholesterol levels and small HDL particles, and increased LDL particle size (Zunino *et al.*, 2012). Two studies in subjects with the metabolic syndrome found that strawberry intervention (50 g/day) for 4–8 weeks led to significant reductions in total (5%

and 6%) and LDL (6% and 11%) cholesterol concentrations; however, only the latter study reported any effects on inflammatory biomarkers, such as decreased vascular cell adhesion protein VCAM-1 (Basu *et al.*, 2009, 2010b). Recently, the same group conducted a dose–response trial, in which a low- or high-dose strawberry beverage (78/155 mg ANC per day) was administered to 60 adults with abdominal adiposity and elevated serum lipids (Basu *et al.*, 2014). After 3 months, the high-dose group showed significant reductions in total and LDL cholesterol compared with the control diet or low strawberry dose. For both the high- and low-dose groups, no effects on adiposity, blood pressure, blood glucose, HDL cholesterol, triglycerides, CRP, or adhesion molecules were observed. In a study of healthy volunteers, 500 g daily strawberry supplementation for 1 month resulted in significant reductions in total and LDL cholesterol concentrations and triglycerides (Alvarez-Suarez *et al.*, 2014).

An intervention trial involving participants with type 2 diabetes mellitus (T2D) reported that strawberry significantly reduced CRP levels and lipid peroxidation (Moazen *et al.*, 2013). In contrast, a more recent trial administered a strawberry beverage or placebo to subjects with T2D daily for 6 weeks and observed that diastolic blood pressure was the only parameter in which the reduction was significantly different between treatment groups (Amani et al., 2014). Lower cumulative plasma concentrations of interleukin (IL)-6 and hs-CRP were observed after acute intake of strawberry powder (10 g) compared with placebo in overweight adults (Edirisinghe *et al.*, 2011). A follow-up study using the same participants examined the effects of chronic intake on postprandial inflammatory response (Ellis *et al.*, 2011). After 6 weeks of daily strawberry intake, postprandial plasminogen activator inhibitor-1 (PAI-1) and IL-1b were significantly decreased, although in contrast to the previous study, IL-6 and hs-CRP were not different between the strawberry and placebo treatments.

### *1.5.2. Cyanidin (chokeberry, raspberry, cherry, purple corn and purple carrot as major sources)*

Cy-3-glc undergoes rapid and extensive degradation and/or metabolism after ingestion. Interestingly, there is evidence that Cy may undergo methylation to form Pn in vivo, as indicated by studies with humans and rats (Tsuda *et al.*, 1999; Wu *et al.*, 2002; Vanzo *et al.*, 2011). Orally administered Cy-3-glc in rats led to eightfold higher plasma concentrations of PCA compared with the parent molecule, indicating PCA to be the major metabolite in vivo (Tsuda *et al.*, 1999). PCA was confirmed as the major human metabolite of Cy-3-glc after ingestion of blood orange juice, accounting for ~44% of ingested Cy-3-glc in the plasma and ~73% of Cy-3-glc excreted in the urine (Vitaglione *et al.*, 2007). An in vitro study revealed that, after 4 h at physiological pH, 96% of Cy and 56% of Cy-3-glc degraded into PCA and 2,3,6-trihydroxybenzaldehyde (Kay *et al.*, 2009). PCA was similarly the major metabolite of Cy-3-glc after incubation with gut microflora, which catalyzed the hydrolysis both of Cy-3-rut to Cy-3-glc and of Cy-3-glc to Cy aglycone, ultimately forming PCA (Aura *et al.*, 2005). These results indicated that the intestinal deglycosylation of Cy-3-glc, either pH mediated or by bacteria, facilitates its degradation to phenolic acids, primarily PCA. It has been further shown in vitro that human liver microsomes metabolized Cy to PCA, which was then further metabolized to form three glucuronide conjugates of PCA (Woodward *et al.*, 2011).

Studies in which [$^{13}$C]Cy-3-glc was administered to humans indicated that the major metabolites of Cy-3-glc ingestion in humans are the phenolic acids resulting from Cy-3-glc degradation, including benzoic, phenylacetic, and phenylpropanoic acids, phenolic aldehydes, and hippuric acid, in addition to their phase II conjugates (Czank *et al.*, 2013; de Ferrars *et al.*, 2014). Anthocyanin conjugates (Cy-3-glu and methylated and glucuronidated conjugates of Cy-

3-glu or Cy) were also observed, but at concentrations that were 60- and 45-fold lower than those of the phenolic metabolites in plasma and urine, respectively (de Ferrars *et al.*, 2014). Czank *et al.* (2013) demonstrated that orally administered Cy-3-glu (500 mg) had a relative bioavailability of 12.38% (5.4% excreted in urine and 6.9% in breath) in human volunteers, far higher than had been estimated previously.

Acylated Cy-based ANCs, such as from purple carrot, are extremely poorly bioavailable and do not appear to be significantly metabolized in vivo. In a clinical feeding study using purple carrot ANCs, acylated Cys were absorbed and excreted intact, but were detected at 11–14-fold lower levels in urine and 8–10-fold lower levels in plasma relative to non-acylated ANCs (Kuriliche *et al.*, 2005). Similar results were found in human feeding studies, indicating that acylated Cys from purple carrot are not metabolized upon ingestion (Charron *et al.*, 2009; Novotny *et al.*, 2012).

Cy, particularly Cy-3-glc, has been extensively studied for their potential role in the treatment and prevention of obesity and related metabolic disorders. Cy-3-glc has demonstrated moderate antidiabetic, anti-inflammatory, and cholesterol-regulatory effects in vitro and in animal models. Tsuda *et al.* (2003) first reported that Cy-3-glc (from purple corn) ameliorated the development of insulin resistance and obesity in C57BL/6 J mice; after 12 weeks of HFD, mice on the control diet developed hyperinsulinemia, hyperglycemia, and hyperleptinemia, all of which remained normal in mice receiving Cy-3-glc. Furthermore, Cy-3-glc treatment suppressed the HFD-induced increases in tumor necrosis factor alpha (TNF-α) expression, inhibited the expression of enzymes involved in fatty acid and triacylglycerol synthesis, and reduced SREBP-1 expression in white adipose tissue (Tsuda *et al.*, 2003). In diabetic KK-A(y) mice, Cy-3-glc significantly reduced blood glucose and enhanced insulin sensitivity in mice with T2D by

upregulating GLUT4 and downregulating RBP4 in white adipose tissue (Sasaki *et al.*, 2007). Dietary supplementation with Cy-3-glc for 5 weeks reduced fasting blood glucose and improved insulin sensitivity, lowered levels of white adipose tissue mRNA and serum inflammatory adipocytokines (TNF-α, IL-6, MCP-1), inhibited macrophage infiltration in adipose tissue, and ameliorated hepatic steatosis and triglyceride concentrations in both HFD-fed C57BL/6J mice and diabetic db/db mice (Guo *et al.*, 2012).

Adipocyte dysfunction represents a major therapeutic target for Cy-3-glc; the mechanisms involved in the anti-obesity therapeutic effects of ANCs in general are outlined in Azzini, Giacometti, & Russo (2017). In vitro studies with rat or human adipocytes have indicated that Cy-3-glc enhances the expression and secretion of adiponectin and leptin, increases PPARγ expression, upregulates genes involved with lipid metabolism, including hormone sensitive lipase and acyl-CoA oxidase 1 (ACOX1), and downregulates the expression of inflammatory mediators such as plasminogen activator inhibitor-1 (PAI-1) and IL-6 (Tsuda *et al.*, 2004, 2005, 2006). The enhancement of adiponectin secretion by Cy-3-glc involves modulation of the JNK-forkhead boxO1 (Foxo1) signaling pathway (Guo *et al.*, 2008, 2012). Liu *et al.* (2014a) showed that, in human subjects with T2D, Cy-3-glc treatment restored diabetes-related endothelial dysfunction via enhanced adiponectin expression and secretion, which increased endothelial NO bioavailability. It has also been suggested that Cy-3-glc affects glucose metabolism through modulation of AMPK. Kurimoto *et al.* (2013) showed that Cy-3-glc (from black soybean seed coat extract) ameliorated hyperglycemia and insulin sensitivity via the activation of AMPK in T2D mice. AMPK activation was observed in skeletal muscle and liver and was accompanied by upregulated GLUT4 in skeletal muscle and downregulated hepatic gluconeogenesis. In vitro

investigation with L6 myotubes revealed that Cy-3-glc stimulated AMPK activation, which enhanced cellular glucose uptake (Kurimoto *et al.,* 2013).

Anti-inflammatory and lipid-lowering effects have been shown in several studies. Cy-3-glc inhibited IL-8, IL-6, and MCP-1 release dose dependently in human endothelial cells stimulated with CD40 via inhibition of NF-κB signaling (Xia *et al.,* 2007). Similarly, Sasaki *et al.* (2007) observed that Cy-3-glc suppressed MCP-1 and TNF-α expression in mice with T2D. Cy-3-glc dose dependently induced cholesterol efflux in mouse peritoneal macrophages via activation of a signaling pathway involving PPARγ, LXRα, and ABCA1 (Xia *et al.*, 2005). Activation of liver X receptor alpha (LXRα) appears to be crucial for the anti- inflammatory effects of Cy-3-glc (Wang *et al.*, 2008), and Cy-3-glc and Cy were recently shown to decrease cholesterol accumulation and inhibit the production of MCP1, ICAM1, and TGFβ1, via activation of not only PPARα but also LXRα in HK-2 cells (Du *et al.*, 2015).

Several studies have also demonstrated that the major Cy-3-glc breakdown metabolite, PCA, itself exhibits significant biological activity. Scazzocchio *et al.* (2011) demonstrated that both Cy-3-glc and PCA exerted insulin-like activities in human omental adipocytes and 3T3-L1 cells through PPARγ activation, resulting in the upregulation of adiponectin and GLUT4. PCA and Cy-3-glc demonstrated potent anti-inflammatory effects (as measured by suppressions of TNF-α, IL-1β, COX-2, NO, PGE2 , and iNOS) in vitro in RAW 264.7 cells and in vivo in BALB/c mice through inhibition of NF-κB and MAPK activation (Min *et al.*, 2010). In contrast with this result, Serra *et al.* (2013) reported that Cy-3-glc reduced NO, PGE2, and IL-8 production and inhibited iNOS and COX-2 expression through a mechanism that involved the suppression of STAT1 activation but did not significantly involve NF-κB or MAPK activation. Wang *et al.* (2011) demonstrated that PCA inhibited monocyte adhesion and NF-κB activation in

vitro in TNF-α-activated mouse aortic endothelial cells, and furthermore attenuated atherosclerosis development in ApoE-deficient mice. Hidalgo *et al.* (2012b) observed that high concentrations of PCA modestly inhibited NO production and TNF-α secretion in LPS-INF-γ-induced macrophages. PCA also inhibited LDL oxidation in vitro in murine J774 A.1 macrophages (Masella *et al.*, 2004; Vari *et al.*, 2015).

Chokeberry intake lowered the concentrations of blood glucose, triglycerides, and total and LDL cholesterol in rats fed a fructose-rich diet for 6 weeks (Qin and Anderson, 2012). Plasma adiponectin levels were increased and plasma TNF-α and IL-6 were inhibited by chokeberry compared with controls, and both protein and gene expression of adiponectin and PPARγ mRNA levels were upregulated after chokeberry intake.

Chokeberry extract has been also investigated in several clinical trials. In a study with individuals who had survived a myocardial infarction and were receiving statin treatment for at least 6 months, 255 mg/day of a chokeberry extract (25% ANCs) in combination with statin treatment resulted in significant reductions in hs-CRP, IL-6, sICAM-1, sVCAM-1, and MCP-1 after 6 weeks (Naruszewicz *et al.*, 2007). In two studies involving subjects with the metabolic syndrome, chokeberry extract (providing at least 60 mg ANCs per day) for 1–2 months led to significant reductions in blood pressure, triglycerides, and total and LDL cholesterol (Broncel *et al.*, 2010; Sikora *et al.*, 2012). In another study, 200 ml of chokeberry juice daily for 4 weeks significantly reduced systolic and diastolic blood pressure and triglyceride levels in hypertensive subjects; it also tended to reduce total and LDL cholesterol concentrations, although these effects did not reach statistical significance. Overall, the results indicate that chokeberry extract may improve blood pressure and lipid status, especially under chronic disease conditions.

Raspberries were shown to decrease COX-1 and COX-2 activities in vitro (Seeram *et al.*, 2001). Li *et al.* (2014) evaluated the anti-inflammatory effects of ANC-rich fractions from red raspberries in RAW264.7 macrophages and subsequently in a mouse model of colitis. In LPS-stimulated macrophages, ANC-rich red raspberry fractions efficiently inhibited NO production and attenuated expression levels of iNOS, COX-2, IL-1β, and IL-6 via modulation of NF-κB and MAPK/JNK/AP-1 signaling pathways. ANC-rich red raspberry fractions also abated dextran sulfate sodium (DSS)-induced weight loss and histological damage in a mouse colitis model.

Prior *et al.* (2009) reported that whole powdered black raspberry supplementation did not prevent the development of obesity or improve lipid status in C57BL6J mice receiving HFD. The same group also tested the effects of supplementation either with black raspberry juice or with purified ANCs from black raspberry on obesity development; neither treatment significantly affected serum cholesterol, triglycerides, or MCP-1 of mice fed an HF60 diet, or altered the development of obesity (Prior *et al.*, 2010a,b). It was suggested that the di- and triglycosidic nature of black raspberry ANCs obviated their ability to modulate obesity.

However, several studies have provided support for the anti-inflammatory effects of black raspberries. In colorectal cancer patients receiving black raspberry powder (1669 mg ANCs per day), all patients who received treatment for at least 10 days (up to 9 weeks) exhibited significant reductions in plasma granulocyte macrophage colony stimulating factor (GM- CSF) and IL-8 (Mentor-Marcel *et al.*, 2012). Sardo *et al.* (2016) examined the effects of black raspberry powder on the postprandial inflammatory response in overweight participants. The postprandial test involved the co-ingestion of 45 g of black raspberry powder with a high-fat/ high-carbohydrate meal; prior to the postprandial test, participants consumed 45 g of black raspberry powder for four consecutive days. Compared with the control subjects, a significant

decrease in the area under the curve (AUC) for IL-6 was noted after black raspberry intake; however, no effects on TNF-α or CRP were found (Sardo *et al.,* 2016). Two studies evaluated the effects of black raspberry extract (750 mg ANCs per day) for 12 weeks on lipid status and inflammatory mediators (Jeong *et al.,* 2014, 2016). In participants receiving black raspberry, significantly greater decreases from baseline were observed in total cholesterol levels, ratio of total cholesterol to HDL cholesterol, IL-6, and TNF-α. An ANC-rich fraction of *Rubus coreanus* (containing Cy aglycone, Cy-3-glc, and Cy-3-rut at ratios of 0.44:1.26:0.56 μg/mg fraction) reduced LPS-induced iNOS protein expression and attenuated both mRNA and protein expression of TNF-α, IL-6, and IL-1β via inhibition of MAPK and STAT3 phosphorylation in RAW264.7 macrophages (Jo *et al.,* 2015). A mixture of purified ANCs that contained the same ratios of Cy, Cy-3-glc, and Cy-3-rut also achieved similar anti-inflammatory effects. *R. coreanus* extract inhibited oxLDL uptake in murine macrophages in vitro through the inhibition of JNK phosphorylation (Bhandarye *et al.,* 2012).

Cherries have demonstrated potent inhibitory effects against lipid peroxidation and inflammation (COX enzyme activity) in cell cultures (Wang *et al.*, 1999; Seeram *et al.*, 2001; Šarić*et al.*, 2009). It has been suggested that tart cherry may offer protection against obesity and the metabolic syndrome. In Dahl salt-sensitive rats with insulin resistance and hyperlipidemia, 90 days of dietary supplementation with tart cherries was associated with reductions in fasting blood glucose, hyperlipidemia, hyperinsulinemia, and hepatic steatosis; increases in hepatic PPARα mRNA and in PPARα target acyl-coenzyme A oxidase mRNA and activity were also observed (Seymour et al., 2008). In obese Zucker rats, tart cherry intake for 90 days reduced hyperlipidemia, fat mass, and abdominal adiposity, significantly attenuated plasma and retroperitoneal IL-6 and TNF-α expression and inhibited NF-κB activation, and increased

retroperitoneal PPARα and PPARγ mRNA (Seymour *et al.*, 2009). Tart cherry was associated with reduced post-exercise oxidative stress and inflammation in trained human subjects (Howatson *et al.*, 2010; Bell *et al.*, 2014). However, Lynn *et al.* (2014) reported that tart cherry consumption for 6 weeks did not significantly improve CRP, total and HDL cholesterol, or sICAM-1 concentrations in healthy adults.

There is more robust evidence from clinical trials supporting the anti-inflammatory properties of sweet cherries. In healthy adults, consumption of 280 g/day of Bing sweet cherries for 28 days selectively modulated circulating concentrations of CRP, NO, and RANTES, but did not affect plasma lipid profile, fasting blood glucose or insulin concentrations (Kelley *et al.*, 2006). In a follow-up study with administration of the same dosage, sweet cherry intake was associated with significantly reduced circulating concentrations of CRP, extracellular ligand for the receptor for advance glycation end products, ferritin, PAI-1, endothelin-1, EGF, and IL-18, and increased IL-1 receptor antagonist (Kelley *et al.*, 2013). Taken together, the available evidence indicates that cherries, especially sweet cherries, might confer potent anti-inflammatory effects, but the effects of cherries on metabolic risk factors remain controversial.

Cornelian cherry has also been implicated in the management of diabetes and obesity in several studies. Jayaprakasam *et al.* (2006) reported that dietary enrichment with Cornelian cherry ANCs for 4 weeks ameliorated obesity and insulin resistance in C57BL/6 mice fed HFD. Furthermore, a purified ANC mixture from Cornelian cherry (containing Cy-3-gal, Pg-3-gal, and Dp-3-gal) decreased body weight gain, decreased lipid accumulation and triacylglycerol concentrations in the liver, improved glucose intolerance, and elevated insulin levels (Jayaprakasam *et al.*, 2006). Recently, a clinical trial demonstrated that daily consumption of Cornelian cherry extract (600 mg ANCs per day) improved glycemic control by increasing

insulin concentrations and decreasing triacylglycerol concentrations in the serum of persons with T2D (Soltani *et al.*, 2015).

Poudyal *et al.* (2010) suggested that purple carrot might be effective in the treatment of metabolic syndrome. In a rat model of the metabolic syndrome induced by HF/HC diet, 8 weeks of purple carrot juice intake attenuated abdominal fat deposition, improved glucose tolerance and lipid status, normalized circulating concentrations of CRP and oxidative stress markers (malondialdehyde, uric acid), and protected the liver from structural and functional changes associated with NAFLD. In contrast, a clinical study of purple carrot in overweight and obese participants with normal lipid and inflammatory statuses revealed that purple carrot consumption (118.5 mg ANCs per day) for 4 weeks had no significant effect on CRP, body weight/composition, LDL and total cholesterol levels, or blood pressure; HDL cholesterol levels were modestly but significantly lower in the purple carrot group compared with the placebo group (Wright *et al.*, 2013).

*1.5.3. Delphinidin (blackcurrant, maqui berry and Concord grape as major sources)*

Wu *et al.* (2005) detected only intact Dp glycosides in the urine of pigs following blackcurrant administration, indicating that Dp glycosides may not be significantly metabolized and appear in the plasma and urine in their intact form. Interestingly, a study in rats with administration of a very high dose of pure Dp-3-glc observed 4′-O-methyl-Dp-3-glc in the plasma as the exclusive metabolite, indicating that O-methylation might be a significant route of metabolism for Dp ANCs (Ichiyanagi *et al.*, 2004). In rats, ingested Dp-3-rut was absorbed and excreted primarily intact; minor amounts of 4′-O-methyl-dp-3-rutinoside were observed as the sole metabolite (Matsumoto *et al.*, 2006). Minor amounts of Dp glucuronide were detected in human plasma and urine after ingestion of Concord grape juice (Stalmach *et al.*, 2012).

Dps are very unstable in the GI tract and may not be substantially absorbed in the small intestine in their intact form (Talavera *et al.*, 2004; Yi *et al.*, 2006; Steinert *et al.*, 2008). Steinert *et al.* (2008) tested the GI absorption of blackcurrant ANCs in vitro using Caco-2 cells and found that under acidic conditions, Dps were stable and were more efficiently absorbed than Cys; however, exposure to intestinal pH conditions led to significant chemical degradation and reduced their absorption efficiency compared with Cys. Dp-3-glc also exhibited the lowest absorption efficiency in Caco-2 cells (Yi *et al.*, 2006; Liu *et al.*, 2014b, Kuntz *et al.*, 2015b).

Gallic acid is the major degradation product of Dp, although gallic acid may itself be unstable and subject to further degradation into smaller phenolic products such as pyrogallol, catechol, resorcinol, and phloroglucinol (Forester and Waterhouse, 2008; Hidalgo et al., 2012b). Gallic acid was not recovered in the urine of volunteers after ingestion of a bilberry- lingonberry purée (Nurmi *et al.*, 2009). The ortho-hydroxyl structure of Dp is retained in gallic acid, which may make the latter susceptible to O-methylation in vivo, potentially forming hydroxyvanillic acid and ultimately syringic acid.

Jayaprakasam *et al.* (2005) tested the ability of various anthocyanins to act as insulin secretagogues in rodent pancreatic B cells. After Cy-3-glc, Dp-3-glc was found to be the most effective at stimulating insulin secretion at 4 and 10 mM glucose concentrations. Of the common anthocyanidins, Dp exhibited the greatest inhibitory effects on lipid accumulation and downregulation of PPARγ and Srebp1c mRNA levels in 353-L1 cells (Suzuki *et al.*, 2011). Recently, it was demonstrated in vitro that Dp-3-rut significantly stimulated GLP-1 secretion in vitro in murine GLUTag cells, suggesting a potential therapeutic role for Dp-3- rut in the treatment and prevention of T2D (Kato *et al.*, 2015). Anti-inflammatory effects were also

demonstrated in vitro for gallic acid, which inhibited the release of MCP-1, ICAM-1, and VCAM-1 in endothelial cells (Hidalgo *et al.*, 2012a).

Edirisinghe *et al.* (2011) demonstrated in vitro that blackcurrant extracts protected against endothelial dysfunction via activation of eNOS via the Akt/PI3 kinase pathway in human umbilical vein endothelial cells (HUVECs). In diet-induced obese C57BL/6J mice, blackcurrant extract supplementation reduced obesity-induced inflammation in adipose tissue by upregulating genes involved in energy expenditure and mitochondrial biogenesis (PPARα, PPARδ, UCP-2, UCP-3, and mitochondrial transcription factor A) in skeletal muscle, and additionally by reducing TNF-α and IL-1β mRNA in splenocytes (Benn *et al.*, 2014). The same group recently demonstrated that blackcurrant extract improved hypercholesterolemia, hyperglycemia, and liver steatosis in mice fed an obesogenic diet, likely due to altered energy metabolism in skeletal muscle resulting in increased glucose uptake and energy expenditure (Benn *et al.*, 2015).

In lean and high-fat diet-induced obese C57BL/6J mice with healthy gut microbiome, Esposito *et al.* (2015) reported that supplementation with 1% blackcurrant extract attenuated body weight gain and improved insulin sensitivity, irrespective of the fat content of the diet. These effects were not observed in mice with antibiotic-disrupted gut microbiomes, suggesting that the protective effects of blackcurrant ANCs against obesity development and insulin resistance were mediated by gut microbial metabolites. Lyall *et al.* (2009) examined blackcurrant supplementation in human exercise and in vitro models, and found that blackcurrant inhibited NF-κB activation and the LPS-stimulated secretion of TNF-α and IL-6 in THP-1 cells. Jin *et al.* (2011) reported that the ingestion of 250 ml of 20% blackcurrant juice drink in healthy volunteers had no significant effects on postprandial measures of vascular reactivity, biomarkers of endothelial function, or lipid profile.

In HUVECs, maqui berry juice dose-dependently inhibits intracellular oxidative stress induced by hydrogen peroxide, suggesting a protective role for maqui berry against endothelial dysfunction (Miranda-Rottmann *et al.*, 2002). Schreckinger *et al.* (2010) demonstrated that in RAW 264.7 macrophages, the purified ANC fraction of maqui berry significantly attenuated LPS-induced iNOS expression and nitrite production and inhibited COX-2 and PGE2 expression. Furthermore, maqui berry ANCs were associated with reduced lipid accumulation in 3T3-L1 adipocytes. A recent in vitro study by Reyes-Farias *et al.* (2015) found that maqui berry extract repressed markers of inflammation (NO production, iNOS and TNF-α expression) related to the pathogenic interaction between adipocytes and macrophages, which has been associated with the development of low-grade inflammation that occurs as a consequence of obesity.

The antidiabetic effects of maqui berry, from which Dp-3-sam-5-glc was isolated, were studied in vitro and in vivo using a murine model of T2D (Rojo *et al.*, 2012). Oral administration of maqui berries, and also the purified ANC, lowered fasting blood glucose and improved glucose tolerance in obese, hyperglycemic C57BL/6J mice fed HFD. Both maqui berries and pure ANC decreased gluconeogenesis in rat liver cells and increased glucose uptake in L6 myotubes.

Hidalgo *et al*. (2014) evaluated the effects of a standardized extract of maqui berries (Delphinol; ≥25% Dps and ≥35% total ANCs) on postprandial blood glucose and insulin concentrations in moderately glucose-intolerant human volunteers. Delphinol intake significantly attenuated postprandial blood glucose and insulin after rice consumption relative to placebo. Longer term intervention (4 months) in streptozotocin-induced diabetic rats revealed that Delphinol treatment normalized fasting blood glucose levels in diabetic rats. Analysis of rodent jejunum revealed that the Dp in the extract significantly inhibited SGLT-1 activity, indicating

that Dps from maqui berry modulate glycemia by inhibiting SGLT-1 and thus reduce glucose absorption in the small intestine (Hidalgo *et al.*, 2014).

*1.5.4. Malvidin (bilberry, blueberry and grapes as major sources)*

Mv-3-glc was detected intact in the plasma of human volunteers following ingestion of red wine and red grape juice and recovered at 0.02–0.23% of the initial concentration in urine (Bub *et al.*, 2001; Frank *et al.*, 2003). Ingestion of an ANC extract of red wine led to the detection of primarily intact Mv-3-glc along with lesser amounts of Mv glucuronide in plasma and urine (Garcia-Alonso *et al.*, 2009). Stalmach *et al.* (2012) also detected Mv glucuronide in plasma and urine of human volunteers after ingestion of grape juice. The major phenolic degradation product of Mv-3-glc is syringic acid (Hidalgo *et al.*, 2012b). Mechanistic investigations have indicated that the anti-inflammatory activity of Mvs centrally involves regulation of the NF-κB signaling pathway. In bovine arterial endothelial cells, Mv-3-glc treatment inhibited the inflammatory response via inhibition of NF-κB activation, which suppressed iNOS expression and NO synthesis, COX-2 expression, and IL-6 production (Paixão *et al.*, 2012). In addition, Mv-3-glc demonstrated endothelial protective effects in upregulation of eNOS mRNA, which in turn stimulated eNOS activity and increased endothelial NO production. Decendit *et al.* (2013) showed that Mv-3-glc decreased the gene expression of and consequently inhibited TNF-α, IL1, IL-6, and iNOS-derived NO secretion in activated human macrophages. Huang *et al.* (2014) demonstrated that Mv-3-glc and Mv-3-gal inhibited the TNF-α-induced MCP-1, ICAM-1, and VCAM-1 secretion and IκB degradation in a concentration-dependent manner in endothelial cells in vitro. Mv-3-glc showed greater anti-inflammatory effects than Mv-3-gal, but both significantly decreased IκBα degradation and inhibited nuclear translocation of NF-κB.

Bilberry extracts suppressed lipid accumulation in 3T3-L1 cells and prevented adipocyte differentiation by blocking insulin signaling; mRNA levels of PPARγ and SREBP-1C were downregulated (Suzuki *et al.*, 2011). Takikawa *et al.* (2010) reported that bilberry supplementation for 5 weeks in diabetic mice improved hyperglycemia and insulin sensitivity via AMPK activation in the liver, white adipose tissue, and skeletal muscle. Serum triglyceride and total cholesterol levels were also reduced by bilberry treatment as a consequence of PPARα and acyl-CoA oxidase (ACO) upregulation. Recently, Mykkanen et al. (2014) reported that dietary supplementation with 5% or 10% whole bilberries for 3 months had anti-inflammatory and antihypertensive effects, but had only marginal effects on weight/fat gain, glucose tolerance, insulin resistance, or serum lipids in C57BL mice fed a high-fat diet. The levels of certain pro-inflammatory cyokines (IL-1β, IL-2, IL-7, TNF-α, GM-CSF, and MCP-1) tended to be reduced by bilberry supplementation, although only the reduction in MCP-1 reached statistical significance. IL-15 and IFN-γ were increased by HFD intake, but were reduced to undetectable levels in mice receiving bilberries. The authors speculated that although bilberries might attenuate the development of insulin resistance in the early stages of obesity, they are unable to effect significant improvement once the disease has had time to manifest (Mykkanen *et al.*, 2014). Hoggard *et al.* (2013) demonstrated that acute bilberry ingestion reduced postprandial glycemia and insulin in subjects with T2D. Compared with placebo, ingestion of bilberry extract (equivalent to ~50 g of fresh bilberries) significantly decreased the AUC for both glucose and insulin; GLP-1 and MCP-1 secretion were unaffected. It was suggested that bilberries may modulate glycemic response by reducing intestinal carbohydrate absorption.

The anti-inflammatory effects of bilberry are more defined in the recent literature. Karlsen *et al.* (2010) found that intake of bilberry juice (330 ml) for 4 weeks significantly

decreased plasma concentrations of CRP, IL-6, MIG, and IL-15 in human participants with cardiovascular risk factors. Lehtonen *et al.* (2011) subsequently reported that whole bilberries (100 g fresh) for 33–35 days significantly decreased circulating TNF-α and sVCAM-1 in overweight and obese participants. Interestingly, bilberry intake did not increase adiponectin secretion, which the authors speculated might have been due to the brevity of the observation period. In another study of individuals with metabolic syndrome, addition of bilberry (400 g/day) to the diet significantly lowered serum concentrations of hs-CRP and IL-12; reducing effects were also observed with regard to IL-6 and LPS, although these did not reach statistical significance (Kolehmainen *et al.*, 2012).

There are considerable indications that blueberry consumption may alleviate chronic inflammation and endothelial dysfunction, whereas the evidence for obesity-related metabolic disorders is far less robust. Gavage with ANC-enriched extract from lowbush blueberry (containing primarily Dp and Mv) effectively lowered blood glucose levels within 6 h in diabetic C57b1/6J mice; when the component anthocyanin moieties from the natural blueberry extract were administered separately, only pure Mv-3-glu lowered blood glucose levels by 34% in mice, and Dp-3-glu failed to show hypoglycemic activity (Grace *et al.*, 2009). DeFuria *et al.* (2009) showed that in C57BL mice maintained on HFD, blueberry powder supplementation prevented insulin resistance and hyperglycemia but was not associated with changes in energy intake, metabolic rate, body weight, or adiposity compared with the control high-fat diet without blueberry. Similarly, in a study using obese postmenopausal mice, supplementation with blueberry powder inhibited the development of glucose intolerance and hepatic steatosis induced by a high-fat diet, although body weight and fat mass were not significantly altered with blueberry treatment (Elks *et al.*, 2015). In another study with obese Zucker rats, dietary blueberry

supplementation reduced abdominal adiposity, plasma triglycerides, and fasting glucose in obese Zucker rats fed a low-fat diet; moreover, blueberry intake was associated with increased PPAR activity and transcriptional expression in AAT and skeletal muscle (Seymour *et al.,* 2011). In contrast, a recent study indicated that obese Zucker rats fed an 8% blueberry-enriched diet did not exhibit significant reductions in fasting blood glucose or insulin concentrations, although positive effects on certain markers related to glucose metabolism (plasma glycated hemoglobin GHbA1c, resistin, and RBP4) were observed (Vendrame *et al.*, 2015).

A series of experiments investigated the effects of whole blueberries or purified blueberry ANCs on obesity development in C57BL/6 mice; mice were fed 45% or 60% high-fat diets (HFD-45/HFD-60), either alone or supplemented with 10% whole blueberry powder or purified blueberry ANCs administered through drinking water (Prior *et al.*, 2008). Surprisingly, HF45 mice fed whole blueberry powder exhibited more body weight gain and adiposity, whereas HF60 mice receiving purified blueberry ANCs showed reduced weight gain and adiposity compared with controls. In a follow-up investigation, the same group demonstrated that providing purified blueberry ANCs through drinking water was more effective at preventing obesity than blueberry juice in C57BL/6J mice fed a high-fat diet, although both treatments significantly reduced body weight gain and body fat percentage (Prior *et al.,* 2010a). Both treatments lowered serum leptin concentrations, but purified blueberry anthocyanins also improved B-cell function and attenuated fat deposition to a greater extent than blueberry juice, which modestly ameliorated fat gain compared with high-fat controls. In addition, there is some evidence that blueberries might modulate lipid metabolism and improve dyslipidemia. Vendrame *et al*. (2014) reported that blueberry supplementation significantly reduced plasma total cholesterol and TAG in obese Zucker rats, in addition to increasing expression of PPAR, PPARγ, and ATP-binding cassette

transporter 1 in abdominal adipose tissue (AAT), and decreasing expression of fatty acid synthase and SREBP-1 in the liver and in AAT (Vendrame *et al.*, 2014).

Studies of blueberries in humans indicated protective effects of blueberries against endothelial dysfunction, but produced equivocal results with respect to obesity and insulin resistance. Ingestion of 668 mg of blueberry ANCs twice daily as a smoothie for 6 weeks improved the insulin sensitivity but did not affect the adiposity, energy intake, or inflammatory biomarkers in 32 non-diabetic, obese subjects who were insulin resistant (Stull *et al.*, 2010). However, in adults with the metabolic syndrome, blueberry intake via smoothie twice daily for 6 weeks did not significantly improve blood pressure or insulin sensitivity; however, compared with the placebo group, the blueberry group was associated with significantly improved resting endothelial function, expressed as reactive hyperemia index (RHI) (Basu *et al.*, 2010a). Stull *et al.* (2015) reported that twice-daily ingestion of a blueberry smoothie (total 45 g/day of blueberries; 581 mg of ANCs) for 6 weeks did not significantly affect blood pressure or insulin sensitivity compared with placebo in adults with metabolic syndrome; similarly to the previous study, participants consuming blueberries exhibited significantly greater improvement of endothelial function than those consuming placebo. Although a study by Riso *et al.* (2013) found no effects on vascular endothelial function or NO generation after regular consumption of a wild blueberry drink, another study reported that acute blueberry intake improved endothelial function as measured by flow-mediated dilation in healthy men consuming blueberry smoothies (Rodriguez-Mateos *et al.*, 2013). Endothelial function increased both at 1–2 and at 6 h after consuming smoothies containing 766–1791 mg of blueberry polyphenols. After 1 h of consumption, there was a dose-dependent increase in endothelial function up to the 766 mg ANC intake, followed by a plateaued endothelial function response with the higher doses. No changes

in blood pressure were observed. The improvement in flow-mediated dilatation (FMD) observed at 1–2 h was associated with maximal increases in the plasma concentration of ferulic acid, isoferulic acid, vanillic acid, 2-hydroxybenzoic acid, benzoic acid, and caffeic acid, while the improvement in FMD noted at 6 h was associated with phenolic metabolites, including hippuric, hydroxyhippuric acid, and homovanillic acid. These results suggests that smaller phenolic derivatives of anthocyanins are more likely to have mediated the observed vascular effects, probably by inhibiting NADPH oxidase and thus increasing NO bioavailability (Rodriguez-Mateos *et al.,* 2013). It was shown recently in 48 postmenopausal women with pre- and stage 1 hypertension that daily blueberry intake significantly increased NO and lowered systolic and diastolic blood pressure; furthermore, the antihypertensive effects of blueberry powder grew over the course of treatment, as evidenced by significant group×time interactions, indicating that the protective effects of blueberry on endothelial function may depend on the duration of intake (Johnson *et al.,* 2015).

Blueberries have demonstrated extensive anti-inflammatory activity in a number of studies. Blueberry supplementation attenuated the patterns of inflammatory gene upregulation (TNF-α, IL-6, monocyte chemoattractant protein 1, inducible nitric oxide synthase) observed in control HFD mice (DeFuria *et al.,* 2009). An 8-week feeding study with an 8% wild blueberry diet in obese Zucker rats revealed that blueberry supplementation increased adiponectin concentration and decreased plasma levels of TNF-α, IL-6, and CRP. In the liver, blueberries downregulated expression of IL-6, TNF-α, CRP, and NF-κB; similar downregulation was observed in abdominal tissue, with the exception of CRP, which was unaffected (Vendrame *et al.,* 2013). Esposito *et al.* (2014) demonstrated that an ANC-rich fraction of wild lowbush blueberries suppressed mRNA levels of biomarkers of acute inflammation (COX-2, iNOS, IL-

1β) in vitro in LPS-stimulated RAW 264.7 macrophages; furthermore, it was demonstrated that Mv-3-glc significantly modulated the expression of pro-inflammatory genes. An intervention trial in healthy volunteers, administering 300 mg/ day blueberry ANC extract, observed that plasma concentrations of inflammatory mediators (IL-4, IL-3, IL-8, IFN-α) were significantly attenuated by blueberry ANCs (Karlsen *et al.*, 2007).

In healthy volunteers, ingestion of 12 g of ANCs extracted from red wine (~60% mv-3-glc) resulted in a statistically significant decrease in plasma MCP-1 concentrations (Garcia-Alonso *et al.*, 2009). Khadem-Ansari *et al.* (2010) reported that red grape juice consumption (300 ml/day) was associated with significantly increased plasma HDL cholesterol and apolipoprotein B concentrations. More recently, in a clinical trial Yubero *et al.* (2013) administered 700 mg of grape extract to healthy volunteers over 56 days and observed decreases in total and LDL cholesterol levels. In mildly hyperlipidemic patients, 200 mg/day of red grape seed extract for 8 weeks lowered total, LDL, and oxidized LDL cholesterol concentrations (Razavi *et al.*, 2013). In patients with hypercholesterolemia, 500 ml/day of purple grape juice for 14 days reduced ICAM-1 concentrations but did not affect plasma lipids (Coimbra *et al.*, 2005). Dohadwala *et al.* (2010) reported no effect of Concord grape juice on ambulatory blood pressure in relatively healthy individuals with pre- or stage 1 hypertension. However, modest effects were observed on several secondary endpoints, including increased nocturnal dip in systolic blood pressure and reduced fasting blood glucose.

Grapes have also been suggested to improve metabolic dysfunction. In men with metabolic syndrome, taking daily grape powder for 30 days ameliorated endothelial dysfunction as measured by FMD and reduced systolic blood pressure (Barona *et al.*, 2012a). The same group found that 4 weeks of grape supplementation increased plasma levels of anti-inflammatory

IL-10 and adiponectin, but only in men with metabolic syndrome who did not also have dyslipidemia; similar changes were not observed in dyslipidemic subjects with metabolic syndrome (Barona *et al.*, 2012b). In vitro studies of human adipocytes have demonstrated that grape powder extract attenuates markers of insulin resistance, suppresses TNF-α-induced inflammatory gene expression (IL-6, IL-1B, IL-8, MCP-1, COX-2, TLR-2), and inhibits activation of ERK, JNK, c-Jun, and NF-κB (Chuang *et al.*, 2011). Grape powder extract similarly suppressed the LPS-induced inflammatory response in human macrophages by inhibiting the activation of MAPKs, NF-κB, and c-Jun (Overman *et al.*, 2010). In obese mice, supplementation of a high-fat diet with 3% grape powder for 18 weeks improved glucose tolerance and decreased inflammatory markers in serum and adipose tissues (Chuang *et al.*, 2012).

*1.5.5. Anthocyanin mixtures (mixed sources)*

A number of studies have investigated the effects of a mixture of purified ANCs from bilberry and blackcurrant [predominantly Cy (33%) and Dp (58%) glycosides]. Qin et al. (2009) first demonstrated that ANCs improved lipoprotein concentrations in human subjects. In a 12-week trial of 120 individuals with dyslipidemia, the effects of bilberry/blackcurrant ANC ingestion (320 mg) on lipid profiles were evaluated relative to placebo. ANC ingestion significantly increased HDL cholesterol, lowered LDL cholesterol, and enhanced cellular cholesterol efflux to serum due to inhibition of cholesteryl ester transfer protein (CEPT) activity; subsequent in vitro investigation revealed that Cy-3-glc dose-dependently inhibited CEPT in human HepG2 cells (Qin *et al.*, 2009). Both acute (320 mg of ANCs) and long-term (12 weeks; 320 mg of ANCs per day) ingestion of purified bilberry/blackcurrant ANCs significantly improved flow-mediated dilation in individuals with hypercholesterolemia. The 12-week intervention also increased cGMP and HDL cholesterol levels and lowered sVCAM-1 and LDL

cholesterol levels. The improvement in endothelial function was abolished by co-treatment with NO–cGMP inhibitors, thus demonstrating the involvement of the NO–cGMP signaling pathway in the endothelium-dependent vasorelaxation mediated by ANCs. Participants took four capsules daily, each containing 80 mg of ANCs purified from bilberry and blackcurrant (Zhu *et al*., 2011). In a subsequent study by the same group, 320 mg/day of purified bilberry/blackcurrant ANCs for 24 weeks in subjects with hypercholesterolemia significantly lowered plasma hsCRP, sVCAM-1, IL-1B, and LDL cholesterol, and increased HDL cholesterol compared with the placebo treatment (Zhu *et al.*, 2013). In vitro cell culture assays were used to test Dp-3-glc and Cy-3-glc individually and it was found that both inhibited cytokine-induced CRP expression in HepG2 cells and LPS- induced VCAM-1 secretion in endothelial cells; however, the ANC mixture achieved the strongest inhibition of inflammatory cytokines (Zhu *et al.*, 2013). In a further study, Zhu *et al.* (2014) demonstrated that bilberry/blackcurrant ANCs significantly increased HDL and decreased LDL cholesterol concentrations as a result of improved cholesterol efflux capacity, likely due to increased HDL–PON1 activity. A dose of 320 mg/day of bilberry/blackcurrant ANCs given to individuals with diabetes for 24 weeks significantly decreased serum LDL cholesterol, apo-B-48, apo-C-111, and triglycerides, and also increased HDL cholesterol (Li *et al.*, 2015). ANC treatment improved insulin resistance, reduced fasting plasma glucose, and increased serum adiponectin. In healthy subjects, 3 weeks of bilberry/blackcurrant ANC supplementation (300 mg of ANCs per day) reduced plasma concentrations of several NF-κB-related inflammatory mediators, including IL-8, RANTES, IFN-α, IL-4, and Il-13, compared with placebo (Karlsen *et al.*, 2007).

### 1.6. Recent Updates (2017-2020)

Recent observational evidence from prospective cohort studies have implicated the beneficial association of higher dietary (poly)phenolic flavonoids intake in general, and of higher ANCs intake specifically with beneficial outcomes for glycemic dysregulation across the diabetic spectrum. Jennings *et al.* (2020) showed in cross-sectional analysis of 618 individuals from the PopGen cohort that the equivalent of roughly two servings per day of selected ANC-rich foods (1.8 serving/day of strawberries, blackberries, blueberries, or red currants; as well as ANC-rich red wine) was associated with reduced central adiposity measurements (visceral adipose tissue, VAT), as well as greater gut microbiome species diversity as measured by the Shannon index. Moreover, ANC-rich food consumption was associated with changes in the abundance of specific microbial taxa namely as increases of Clostridiales and Ruminococcaceae and reductions of Clostridium XIVa. Changes in microbial diversity to a significant degree and to a lesser extent changes in microbial abundance (18.5% and 3.5% respectively) independently mediated associations between ANC-rich food consumption and central adiposity as measured by VAT; thus the authors suggested that individual differences in gut microbiome species abundance and richness are important determinants underpinning the relationship between consumption of ANC-containing foods and health benefits in the particular context of visceral adiposity-associated cardiometabolic health (Jennings *et al.*, 2020). A meta-analysis of prospective studies by Xu *et al.* (2018) examined studies of flavonoid intake and T2DM risk, including 300,000 participants in total followed up for periods between 4 and 28 years. The relationship between T2DM risk with general flavonoid intake and ANC intake specifically was estimated using random-effects modelling. In spite of the statistical inequivalence of their comparison due to their generation by pooling results from eight and seven studies, respectively,

it is of interest to note that each demonstrated a similarly significant inverse relationship with T2DM risk represented by estimated relative risks of 89% when comparing highest versus lowest quartiles of intake of either (Xu *et al.* 2018). In a cohort of 2231 pregnant women with gestational diabetes, Gao *et al.* (2020b) employed logistic regression modelling to demonstrate an inverse association between gestational diabetes risk and estimations (by dietary frequency questionnaire) of intake of total polyphenols, flavonoids, as well as of ANCs from fruits such as berries. As measured by oral glucose tolerance tests taken between 24 and 28 weeks of pregnancy, women in the highest quartile of ANC intake exhibited an estimated 62% risk of developing gestational diabetes compared with women in the lowest quartile (17.25 vs 0.85 mg ANCs/day, respectively, from fruit intake). Aside from generally supporting the glycemic benefits of dietary polyphenols, flavonoids, and ANCs intake in women, the results of this prospective analysis specifically suggest an intriguing role for dietary intake of ANC-rich fruit such as berries for prenatal maternal health, targeting the prevention and management of gestational diabetes risk (Gao *et al.* 2020b). Evidence from this prospective study represents important initial support for beneficial modulation by ANCs of gestational diabetes risk, an underexplored topic to date.

Human feeding trials have also recently been conducted with whole berries in addition to various berry-based dietary supplements (i.e. purified extracts or (poly)phenolic concentrates) which each provide crucial insight that strengthens the clinical evidence base surrounding the health benefits of dietary supplementation with berry extracts as well as whole berry consumption. Most relevant to the current work, improvements in CMD biomarkers and MetS risk factors have been demonstrated with supplementation of diverse berry and ANC-rich extract sources and in a range of clinical demographics, both including individuals with CMD risk

factors and in healthy individuals. A number of studies have been conducted to investigate the clinical nutritional impacts of consumption of a wide array of different berries, berry (poly)phenolics, and ANC extracts, on both healthy and CMD-relevant populations; a representative selection will be presented here to illustrate the most recent clinical examples of new additions to the body of scientific literature surrounding the health benefits of berries containing (poly)phenolic ANCs – though it is important to note that, while ANCs are often considered to be the most prominent class of bioactive compounds present in colorful berries, they are far from the only compounds of nutritional and therapeutic relevance (Kay *et al.* 2020). While beyond the scope of this review, a comprehensive review of the bioactive phenolic compounds resulting from berry (poly)phenol consumption is presented by Chandra *et al.*, (2019).

Blueberry consumption impacted regulation of muscle progenitor cell function, although this was tested only in women of different age groups (Blum *et al.* 2020). This is interesting in the context of our recent research exploring the same topic of the impacts of berry-based dietary patterns on regulation of muscle development through genetic regulatory factors connected with myogenesis and muscle stem cell (satellite cell) dynamics (Xiong *et al.* 2019) demonstrating how berry supplemented diets in the C57BL/6J mouse model of high fat diet-induced obesity (DIO) led to changes in skeletal muscle genetic markers which are associated with myogenic regulation and are widely speculated as important mediators of postnatal muscle growth and influence muscle development. Blueberry consumption improved cardiometabolic functional biomarkers in MetS individuals in a 6-month feeding intervention trial that can be considered of seminal importance in demonstrating the therapeutic effects of the regular inclusion of reasonable portions (readily achievable and accessible to most individuals) of blueberries in the diet,

specifically and importantly in the context of individuals already diagnosed with mild to moderate MetS risk factors (Curtis *et al.* 2019). Stote *et al.* (2020) also demonstrated in male type 2 diabetic patients aged between 51 and 75 years old that consumption of freeze-dried whole blueberry for eight weeks produced a considerable beneficial impact on several cardiometabolic health indices, most importantly resulting in significant improvements in concentrations of hemoglobin A1c (HbA1C) and triglyceride in men consuming blueberries twice daily compared with men assigned to the placebo intervention group (Stote *et al.*, 2020).

Grape pomace extract improved postprandial insulin response in healthy individuals (Costabile *et al.* 2019). Chokeberry (Aronia berry*)* juice consumption beneficially impacted body composition, reflected by reduction in body fat and concurrent increase in fat-free mass in healthy males (Bakuradze *et al.* 2019); juice consumption was also linked with improvement in a measure of lipid metabolism (plasma phospholipid composition) in individuals with cardiovascular risk factors (Pokimica *et al.* 2019). Supplementation with both Aronia berry extracts and whole fruit powder improved measures of vascular/endothelial function (flow-mediated dilation; FMD) and modulated gut microbiota composition in healthy male individuals, and these improvements were correlated with increased circulating plasma phenolic metabolites after Aronia consumption (Istas *et al.* 2019). Similarly, bilberry consumption in individuals with MetS risk factors showed benefits on fasting insulin secretion and glycemic regulation, and this correlated with increased serum levels of hippuric acid; a limitation of this study is that hippuric acid is often considered to be a 'promiscuous' dietary phenolic metabolite with origins in numerous phenolic compounds outside of ANCs, but the concept of these studies builds on the same hypothesis contending that increases in circulating concentrations of (poly)phenolic metabolites are responsible for the cardiometabolic health benefits of berry consumption and

ANC intake (Chandra *et al.* 2019). The best illustration of this point may come from a recent clinical trial by Rodriguez-Mateos *et al.* (2019), in which daily wild blueberry consumption over the course of one month corresponded with dose-dependent improvement of endothelial function (as measured by FMD) in healthy individuals and also reduced 24-hour ambulatory systolic blood pressure, effects which were highly correlated with specific ANC-derived plasma phenolic metabolites in circulation after both acute and chronic consumption. In the same study, peripheral blood mononuclear cells of mice injected with ANC-derived plasma metabolites exhibited gene expression changes indicating significant regulation of signaling pathways regulating cell adhesion, differentiation, and migration, as well as immune response (Rodriguez-Mateos *et al.* 2019). The latter result suggests alteration by wild blueberry ANCs in cellular signaling pathways controlling immune cell dynamic responses to morphological and immunological challenges. Perhaps most interestingly from the perspective of our own research collaborations, the results of Rodriguez-Mateos *et al.* (2019) builds on and corroborates the results of our recently published *in vitro* investigation in which we demonstrated that incubation of adult human dermal fibroblasts (HDFa), skin cells which are crucial mediators of wound healing and which are centrally involved in repair and regeneration of skin and provide insight into the same processes relevant to other connective tissues (Esposito *et al.* 2019). Incubation of HDFa cultured cells with ANCS and complex (poly)phenolics, especially proanthocyanidins, as well as with specific phenolic catabolites thereof, led to gene expression changes and enrichment in cellular pathways controlling regulation of extracellular matrix (ECM) composition and ECM component (collagen) turnover, and altered signaling processes linked to cell-ECM dynamics and cellular reception of physical changes to surrounding ECM (mechanical signaling; mechanosensitivity), most particularly suggested by modified expression levels of integrin

signaling proteins. Subsequent experiments demonstrated correlation of these changes with alterations in cellular bioenergetics, especially improvements in ATP production (Esposito *et al.* 2019). Thus, it is likely from the results of these two investigations that further research on the impact of wild blueberry ANCs and similar (poly)phenolics as well as their catabolic breakdown products is warranted within the context of their impacts of cellular morphology, immune response, and mechanosensitive interactions between cells and ECM such as those regulated by integrin signaling, which are highly implicated in the effectiveness of connective tissue repair and regenerative functions. These would be important research directions with downstream implications for many biomedical research fields, such as for wound healing and regenerative medicine applications.

Recently, a slew of clinical trials with the proprietary ANC supplement Medox (Biolink Group AS, Sandnes, Norway), comprising encapsulated standardized compositions of purified bilberry and blackcurrant ANCs, have provided robust evidence in support of the therapeutic benefits of purified ANCs in supporting cardiometabolic health and influencing CMD risk factors. The benefits of purified ANC supplementation on cardiometabolic biomarkers were demonstrated in a range of CMD-relevant patient populations, including individuals with prediabetes and early untreated and/or newly diagnosed diabetes (Yang *et al.,* 2017; Yang *et al.* 2020a), fasting hyperglycemia (Yang *et al.* 2020b), dyslipidemia (Xu *et al.* 2020; Zhang *et al.* 2020) as well as in healthy individuals (Guo *et al.* 2020). For example, Xu *et al.* (2020) examined the effects of escalating dosages of purified ANCs (placebo, 40, 80, or 320 mg/day) on lipid profile of dyslipidemic individuals aged 35-70. Compared with those assigned to placebo supplementation, individuals in the 320 mg/day intervention arm showed significant improvements in cholesterol metabolism (cholesterol efflux capacity) and lipid profiles (HDL

cholesterol and apoliprotein A-I concentrations); supplementation with 80 mg/day ANCs was associated with improved HDL cholesterol levels and cholesterol efflux but to a lesser extent. Overall, the results indicated a clear dose-dependent relationship between purified ANC intake and cholesterol efflux capacity in this clinical population lending support to a therapeutic impact of ANC supplementation on lipid homeostasis, suggesting a role for ANCs in the management of dyslipidemia, hypercholesterolemia and related disorders of lipid metabolism that are closely linked with the development of obesity-associated CMDs like T2DM (Cho *et al.* 2019).

**1.7.Conclusions**

The most recent data from preclinical and human studies demonstrate that delphinidin- and malvidin-based ANCs, at concentrations that are attainable in human tissues, are more effective at improving key nutrigenomic targets associated with metabolic and immune health. These targets are central to the regulation of, among others, transcriptional and cytokine-mediated inflammatory signal networks crucial in long-term metabolic health and disease. However, higher gastrointestinal tissue levels of ANCs in animals with antibiotic-disrupted microbiome do not correlate with metabolic health outcomes, possibly indicating that not intact ANCs but their bioactive metabolites are critically responsible for these effects. More clinical trials of similar design with larger populations and of longer duration are necessary to build on this critical evidence, which can be considered as a preliminary basis of support for the effectiveness of blueberry-based nutritional therapies as food-based medical interventions for the clinical treatment (as opposed to prevention) and reduction of disease burden associated with progressive chronic CMDs – a field of rapidly emerging importance as outlined by the NIH Strategic Plan for Nutrition Research 2020-2030 (NIH Nutrition Research Task Force 2020).

Due to heterogeneity in their mechanisms of action that has been speculated for different ANCs based on structural differences, it is appropriate to speculate that the administration of multiple ANCs, as may occur with the ingestion of supplements prepared from berry extracts or of whole berries, may produce the highest degree of beneficial physiological activities due to potential synergistic and/or additive effects of the different structures targeting more therapeutic mechanisms than is possible with individual administration of specific ANCs. Still, in specific instances, these combinatorial interactions may theoretically result in decreased biological efficacy by having antagonistic effects, so little can be said for sure at this point until human feeding trials have been conducted to demonstrate their effects more clearly. Importantly, most ANC-rich berries as well as fruit or vegetable ANC sources generally exhibit mixed ANC profiles, with high degrees of variation in not only their composition (the major type(s) of aglycone present, extent of their structural modifications as glycosylation or acylation), but also in their contents (concentrations of individual ANCs present). Altogether, there is evidence to suggest a therapeutic role for ANC supplementation and ANC-rich fruit or vegetable consumption with regard to cardiometabolic health outcomes - glycemic regulation, insulin sensitivity, lipid metabolism, weight gain, obesity, and body composition, and on the chronic low-grade inflammatory activation which is increasingly understood as a common risk factor and potential pathophysiologic driver of all of the above variables.

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# CHAPTER 2: METABOLIC EFFECTS OF BERRIES WITH STRUCTURALLY DIVERSE ANTHOCYANINS

## 2.1. Introduction

Obesity development and diabetes risks are intimately linked through alterations in insulin signaling and glucose metabolism (Kahn, Hull, & Utzschneider 2006.) The metabolic stressors are often underpinned by physiological changes which ultimately lead to the development of insulin resistance, hyperglycemia, and chronic low-grade inflammation in key metabolic as well as gastrointestinal tissues (Manco, Putignani, & Bottazzo 2010). Previous studies in animal models as well as in humans indicated that dietary supplementation with anthocyanin-containing foods influenced many cardiovascular and metabolic outcomes (Tsuda 2016). These findings were supported by epidemiological observations (Jennings *et al.* 2014) and randomized controlled trials using berries (Huang *et al.* 2016; Stull *et al.* 2010) or mixtures of partially purified anthocyanins (Li *et al.* 2015). However, not all results were consistent between the studies (Van Dam, Naidoo, & Landberg 2013). While berries with primary malvidins and delphinidins generally improved metabolic and cardiovascular disease risk biomarkers (Hansen *et al.* 2005; Karlsen *et al.* 2007; Stull *et al.* 2010; Zhu *et al.* 2011), cyanidins offered little or no protection in many cases (Curtis *et al.* 2009, Giordano *et al.* 2012, Wright *et al.* 2013). Previously, we observed that blackcurrant delphinidins were also more likely to confer favorable metabolic adaptations compared to cyanidins in the diet-induced obese (DIO) mouse model (Esposito *et al.* 2015). Taken together, these findings raised the question of the major differences between various anthocyanins with respect to structure and associated bioactivity.

Anthocyanins are present in plants in different concentrations and with various degrees of hydroxylation, methylation, glycosylation, and acylation (Tsuda 2012). Gastrointestinal metabolism of the primary anthocyanins partially explained a previous perception of their

extremely poor bioavailability after oral administration (Czank *et al.* 2013). The majority of dietary anthocyanins were not absorbed at the upper gastrointestinal level and reached the intestinal microbiome that biotransformed them into phenolic metabolites (Faria *et al.* 2014). Parent aglycone structures and secondary modifications were critical to their stability, bioavailability, and transformation in the gastrointestinal lumen. The in vivo conversion of hydroxylated anthocyanins to their respective methylated counterparts (i.e., malvidins) via Phase II methylation reactions was necessary for enhanced tissue uptake and bioactivity (Prior & Wu 2006) while glycosylation and acylation were associated with increased stability and reduced bioactivity (Charron *et al.* 2009; Kuntz *et al.* 2015).

This important gap in knowledge on the effect of structural differences of the primary anthocyanins and their biological activities represents a major hindrance to achieving consensus over the efficacy and the most effective strategy of dietary supplementation with anthocyanin-rich foods to achieve favorable metabolic and cardiovascular outcomes. The present study was therefore designed to examine changes in metabolic risk factors after consumption of six berries with structurally diverse anthocyanin profiles. Primary anthocyanins were identified in feces of the DIO mice with intact and disrupted gut microbiome and correlated to several measures of metabolic health, including body composition, hyperglycemia, insulin resistance, microbiome profile, and gastrointestinal luminal oxygen and oxidative stress.

## 2.2. Materials and Methods

### *2.2.1. Chemicals*

The cyanidin-3-O-β-glucoside (HPLC grade standard) was purchased from Polyphenols Laboratories AS (Sandnes, Norway). All other chemical reagents and solvents were purchased from Sigma (St. Louis, MO, USA). Commercial whole freeze-dried berry powders were BB,

blackberry (Nubeleaf, Portland, OR, USA); BC, blackcurrant (Just the Berries, Palmerston North, New Zealand); BR, black raspberry (BerriHealth, Corvallis, OR, USA); BL, blueberry (Wild Blueberry Association of North America, Old Town, ME, USA); MB, maqui berry (Sunburst Superfoods, Thornwood, NY, USA). Whole Concord grape puree was purchased from AgriAmerica (Silver Creek, NY, USA) and freeze-dried to obtain CG powder. A commercial blackcurrant powdered extract ACE30 (Active Cassis Extract 30) was kindly provided by Eddie Shiojima (Just the Berries, Palmerston North, New Zealand).

*2.2.2. Animals and Diets*

All animal experiments were approved by the North Carolina Research Campus Institutional Animal Care and Use Committee (IACUC protocols 12-018 and 16-011) in the David H. Murdock Research Institute, the AAALAC accredited animal care facility. Male, 6-week-old C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed four animals per cage under controlled temperature (24 ± 2 °C) and light (12 h light–dark cycle, lights on at 7:00 a.m.). Immediately upon arrival, animals were allowed to adapt to new conditions for 7 days and handling the animals was performed daily during this time to reduce the stress of physical manipulation. Mice were then randomized into ad libitum access to Research Diets (New Brunwick, NJ, USA) low 10 kcal % fat diet D12450J (low fat diet, LFD, 3.85 kcal/g, n = 12) or high 60 kcal % fat diet D12492 (high fat diet, HFD, 5.24 kcal/g, n = 56) and tap water for 6 weeks. Obese mice were further randomized to control high fat diet (HFD, n = 8) or berry-supplemented treatment groups normalized to 400 μg/g total anthocyanins (berry powders incorporated into HFD by Research Diets). Mice were kept on the respective diets for an additional 12 weeks. Animal weight and food intake (accounting for spillage) were recorded weekly for the duration of the study. All animal diets were kept at −80 °C for long-term storage

and stability, and freshly thawed food was dispensed to animals every 3–4 days to limit phytochemical degradation in food matrix. Body composition analysis was performed on unanesthetized mice using EchoMRI (Echo Medical Systems, Houston, TX, USA) during Week 1 and Week 12 of the study.

*2.2.3. Antibiotic Knockdown of Endogenous Gut Microbiome*

An antibiotic cocktail (0.5 g/L vancomycin, 1 g/L neomycin sulfate, 1 g/L metronidazole, 1 g/L ampicillin) previously shown to be sufficient to deplete all detectable commensal bacteria (Wang *et al.* 2011) was administered in drinking water ad libitum to all animals for 1 week (between Week 8 and 9 of HFD treatment).

*2.2.4. Oral Glucose and Insulin Tolerance Tests*

For oral glucose tolerance test, mice were fasted overnight (16 h) and received oral gavage of d-glucose (1.5 g/kg body weight). For insulin tolerance test, mice were fasted for 4 h and received intraperitoneal injection of insulin (0.75 U/kg body weight, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blood glucose concentrations were measured at 0, 15, 30, 60 and 120 min after glucose or insulin challenge in blood samples obtained from tail-tip bleedings, using a glucometer (Lifescan, Johnson and Johnson, New Brunswick, NJ, USA).

*2.2.5. Sample Collection and Oxygen Measurements*

At the end of the experiment, mice were euthanized and blood was collected by heart puncture. Oxygen levels in freshly dissected digestive tracts were measured with 0.3 mm Clark-type oxygen microelectrode ET1125, EPU354 isoPod, and Pod-Vu software v5.5.20 (Edaq, Colorado Springs, CO, USA). Electrode was zeroed in water sparged with nitrogen (1 min/mL water) and calibrated in distilled water saturated with air (20.9% oxygen). The accuracy of the microelectrode to reach zero and 20.9% oxygen was rechecked periodically during experiments.

Microelectrode was inserted through a small hole cut into the gut wall and all readings were completed in 15 s intervals within 4 min after dissection. Percent oxygen was measured in the duodenum, ileum, cecum, and colon at ambient temperature (23 ± 1 °C) and local barometric pressure (National Weather Service). Oxygen partial pressure (1 mmHg = 133.322 Pa = 1 torr) was calculated from percent oxygen reading recorded at −800 mV polarization. Liver, fat, muscle, gastrointestinal tissues (stomach, duodenum, ileum, cecum and colon) and luminal digesta were collected and stored at -80 °C to determine the temporal sequence and signaling events that are responsible for changes in physiology and metabolism. Fecal samples from mice were collected, weighed, and pooled by cage at four time points, including Week 4 (1 month on HFD), Week 8 (2 months on HFD, start of antibiotic treatment), Week 9 (end of antibiotic treatment), and Week 12 (3 months on HFD) and stored at -80 °C.

*2.2.6. Gastrointestinal Microbial Profiles*

Genomic DNA was extracted from mouse fecal samples using QIAamp Fast DNA Stool Mini kits (Qiagen, Germantown, MD, USA), quantified using Take3 plate and Synergy H1 microplate spectrophotometer (BioTek, Sunnyvale, CA, USA), and adjusted to 1 ng/µL. Quantitative real-time PCR was performed on an ABI 7500 Fast (Life Technologies, Carlsbad, CA, USA) in a total volume of 20 µL containing 10 µL 2× SYBR Green PCR Master Mix, 1 µL of each primer from GUt low-density array (GULDA) Array (Bergstrom *et al.* 2012), 4.4 µL of nuclease-free water and 3.6 µL of template DNA. The amplification program consisted of 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; and a dissociation curve (95 °C for 15 s, 60 °C for 15 s, then increasing to 95 °C at 2% rate). The mean Ct-value was determined based on a set threshold value of 0.2 and using the automatic baseline correction. Differences in Ct-values for each bacterial target (N0-normalization) were calculated between

those obtained with the universal and target-specific primers and log-transformed. Fold-changes for target amplicons were calculated as the (log 2) ratio of normalized abundances at different time points.

*2.2.7. Anthocyanin Extraction and Quantification*

Anthocyanins were extracted from berry powders and feces using 60% aqueous methanol (1% trifluoroacetic acid) following a previously reported method for rapid analysis of various anthocyanins in rats (He, Magnuson, & Gusti 2005). Total anthocyanins were determined using the pH differential method (Lee, Durst, & Wrolstad 2005) by producing a rapid and reversible structural change (color shift) and quantifying the difference in absorbance at 520 nm using Synergy H1 (BioTek, Sunnyvale, CA, USA).

*2.2.8. HPLC Analysis of Anthocyanins*

Individual anthocyanins in berry powders and feces were quantified by HPLC as described in our previous publication (Zhang *et al.* 2012). Briefly, filtered samples were injected (10 μL) into a 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV–vis diode array detector (DAD), controlled-temperature autosampler (4 °C), and column compartment (30 °C) using a reversed-phase Supelcosil LC-18 column, 25 mm × 4.6 mm × 5 μm (Supelco, Bellefonte, PA, USA). Standard curves were calculated using peak areas at UV of 520 nm as cyaniding-3-O-glucoside equivalents.

*2.2.9. Statistics*

Data were analyzed by one-way ANOVA followed by Dunnett's multiple-range tests using Prism 6.0 (GraphPad Software, San Diego, CA, USA). Body weight gain, glucose and insulin tolerance were analyzed by two-factor repeated-measures ANOVA, with time and

treatment as independent variables. All data were presented as means ± SEM. Significant differences were accepted when the p-value was <0.05.

## 2.3. Results

### *2.3.1. Characterization of Whole Berry Anthocyanins*

The HPLC-DAD analysis was used to characterize and quantify anthocyanins in freeze-dried powders from whole berries and fecal samples (Bergstrom *et al.* 2012; He, Magnuson, & Giusti 2005; Lee, Durst, Wrolstad 2005; Wang *et al.* 2011) (Figure 2.1). The primary anthocyanins in blackberry and black raspberry were mono-glycosylated cyanidins (93%) and di-glycosylated cyanidins (91%), respectively, in good agreement with previously reported anthocyanin profiles for these berries (Dossett, Lee, & Finn 2010; Zhang et al 2012). Blackcurrant and maqui berry contained delphinidins as primary (58% and 78%) and cyanidins as secondary (42% and 21%) anthocyanins, and most pigments were present in the di-glycosylated, non-acylated form as reported earlier (Fredes *et al.* 2014; Slimestad & Solheim 2002). Blueberry and Concord grape had the most complex anthocyanin profiles enriched with malvidins (34% and 7%), petunidins (23% and 19%), and delphinidins (29% and 37%), which were predominantly mono-glycosylated and non-acylated (blueberry) or acylated (Concord grape), as observed previously (Yousef *et al.* 2013; Wang, Race, & Shrikhande 2003). Blueberry (100%) and blackberry (93%) were the best sources of mono-glycosylated anthocyanins, while blackcurrant (84%) and maqui berry (62%) were predominantly di-glycosylated. Acylation was highest in anthocyanins from Concord grape (54%). Major anthocyanins quantified in all samples were listed in the order of elution in Table 2.1 and their compositional differences were summarized in Table 2.2. Freeze dried whole berry powders were then normalized to the total anthocyanin content and incorporated into the high fat diet (HFD) to contain 400 μg/g total

anthocyanins from each berry. Anthocyanin levels in the diet remained stable for at least four days at room temperature and for the duration of the study when diets were stored at -80 °C.

*2.3.2. Changes of Body Weight and Food Intake*

Six-week-old mice were fed LFD or HFD for six weeks to initiate development of obesity in the HFD animals. Next, the HFD treatment groups were randomized to individual berry diets for an additional 12 weeks. There were no abnormal clinical signs throughout the entire study. After 18 weeks on HFD, obese mice developed 68.7% larger body weights as compared to their LFD controls (51.3 g versus 30.4 g, Figure 2.2A). Incorporation of berry powders into the diets for 12 weeks resulted in 6% smaller body weights in animals consuming blackcurrant or Concord grape, and a 3% decrease in body weight of animals treated with blueberry (not significant at $p < 0.05$). Body weight loss was absent in animals consuming blackberry, black raspberry, or maqui berry. Antibiotic cocktail given to animals in drinking water for one week to suppress gastrointestinal microbiome (Week 8–9 on berry diets) resulted in a prominent 10%–15% body weight loss in all LFD and HFD animals ($p < 0.05$).

This effect was significantly attenuated in animals consuming blackberry or maqui berry and correlated to the final body weight gains in these groups (Figure 2.2B). Antibiotic cocktail had a similar effect on food intake of all animals (Figure 2.2C), however all mice recovered their body weights and food intakes once antibiotics were removed from the drinking water. Consumption of Concord grape increased average food intake (13.7%, $p < 0.05$) and this effect was not observed in other treatments.

*2.3.3. Changes in Body Composition*

Berry supplementation had differential effects on body composition of the treated animals. Consumption of blueberry and blackcurrant markedly increased lean body mass (9.8%–

10.2%, $p < 0.05$), water mass (9.9%–10.1%, $p < 0.05$), and concurrently decreased fat body mass in these animals (−17.9%–24.6%, $p < 0.05$), resulting in a non-significant net decrease in body weight gain (Figure 2.3). Supplementation with Concord grape slightly decreased fat body mass, while black berry, black raspberry, and maqui berry supplementation had no significant effect on body composition.

*2.3.4. Anthocyanins in Fecal Samples*

When compared at different time points (4, 8, and 12 weeks on berry diets), anthocyanins were detectable in feces of all treatments groups, albeit at different amounts (Table 2.3). Feeding with blueberry and blackberry resulted in low anthocyanin content of the feces after four weeks of supplementation (2.37–5.30 μg/g), suggesting that most of the ingested parent anthocyanins were absorbed, broken down, and/or metabolized in the gastrointestinal tract. Blackcurrant and maqui berry showed higher resistance to gastrointestinal break down and accumulated in feces in 4–10-fold concentrations as compared to the other berries (26.35–44.93 μg/g). This trend persisted in Weeks 8 and 12 of the study. Addition of the antibiotic cocktail to the drinking water for one week visually intensified red/purple coloration of the feces collected during this time. Disruption of the gastrointestinal microbiome resulted in remarkable accumulation of the parent anthocyanins in feces, with maqui berry, blackcurrant, and Concord grape being most affected (260.58–339.32 μg/g). The enhanced accumulation of anthocyanins was detectable to a lesser degree in black raspberry (120.97 μg/g) and virtually undetectable in blackberry and blueberry (8.51–46.57 μg/g).

*2.3.5. Effect on Glucose Metabolism and Insulin Sensitivity*

Baseline blood glucose levels were measured on Week 12 of berry supplementation after overnight fast (Figure 2.4A). HFD animals showed significantly increased blood glucose levels

as compared to LFD controls (136 vs. 65 mg/dL, $p < 0.01$). Supplementation with blackberry and black raspberry led to a modest increase in fasting glucose (153–164 mg/dL, $p < 0.05$), while the rest of the berries did not affect blood glucose levels. However, berry consumption directly affected glucose and insulin tolerance in these animals. While blood glucose levels of the HFD controls were significantly higher at 30, 60, and 120 min after oral glucose treatment as compared with the LFD mice, animals consuming blackcurrant, blueberry, and Concord grape showed a trend for lower peak glucose concentrations that did not reach significance (Figure 2.4B, 2.4C). These effects were more pronounced in the insulin tolerance test, when animals supplemented with black currants and blueberry showed increased insulin sensitivity similar to the LFD controls (Figure 2.4D, 2.4E).

*2.3.6. Changes in Microbiome and Gastrointestinal Lumen Oxygen*

Gastrointestinal bacterial profiles were strongly affected by HFD and berry diets. Analysis of bacterial relative abundance in fecal samples showed that Firmicutes were the largest group of bacteria in healthy LFD animals (71%) that was further expanded to 89% with HFD diet. This expansion was achieved by the significant decreases in the obligate anaerobe populations of Bacteroidetes and Actinobacteria (Figure 2.5). Berry supplementation with blackberry and black raspberry did not change this trend. Animals consuming Concord grape showed the largest expansion of Actinobacteria from 2% to 18% relative to HFD controls. Blueberry and blackcurrant supplementation was associated with significant shifts in the fecal bacterial profiles that simultaneously increased populations of obligate anaerobes Bacteroidetes (from 7% to 10%–12%) and Actinobacteria (from 2% to 9%–15%). While the mechanism responsible for these shifts are not clear, it is possible that consumption of berries and

specifically anthocyanins reduced oxygen tension in the gut lumens, thus promoting growth of oxygen-sensitive bacterial populations.

To answer this question, we focused on the blackcurrant as this berry was highly enriched with anthocyanins and largely devoid of other polyphenols (Mbeunkui *et al.* 2012). We used 0.3 mm Clark-type oxygen microelectrode to measure pO2 in the four sections of the gastrointestinal lumen including duodenum, ileum, cecum, and colon in animals fed LFD or HFD diet (Figure 2.6A). The oxygen tension gradually decreased along the gastrointestinal tract, suggesting that oxygen entered the gut during feeding and was depleted as the food moved posteriorly. HFD diet was associated with higher oxygen tension in all compartments of the gastrointestinal lumen (Figure 2.6B), with highest differences found in the duodenum (15 vs. 22 mmHg), cecum (4 vs. 6 mmHg) and colon (2.6 vs. 3.6 mmHg). Remarkably, blackcurrant diet reduced luminal pO2 values near to the LFD baseline in all four gastrointestinal compartments tested (Figure 2.6C). This effect could be largely attributed to the anthocyanins present in the blackcurrant, as it was more profound in animals fed HFD supplemented with 1% ACE30 anthocyanin-rich blackcurrant extract that was effective at reducing metabolic risk factors in DIO mice previously (Esposito *et al.* 2015).

**2.4. Discussion**

A single serving of anthocyanin-rich berries may contribute in excess of 100–200 mg anthocyanins to a regular diet (McGhie *et al.* 2003), which is 5–10-fold higher than the daily intake of other flavonoids (Hertog *et al.* 1993). However, anthocyanins from different plants show various degrees of hydroxylation, methylation, glycosylation, and acylation (Tsuda 2012). This may explain the equivocal results from animal and human studies, since predominantly delphinidin- and malvidin-containing fruits are more likely to improve metabolic and

cardiovascular risk factors (i.e., blueberry (Stull *et al.* 2010), black currants and bilberry (Karlsen *et al.* 2007; Zhu *et al.* 2011), or grapes (Hansen *et al.* 2005)), while cyanidins offer less protection (i.e., elderberry (Curtis *et al.* 2009), blood orange (Giordano *et al.* 2012), or purple carrot (Wright *et al.* 2013)).

To understand the relationship between structural constraints and biological activity of anthocyanins in the native food matrix, we selected six berries with diverse anthocyanin compositions for this study (Table 2.1.). Blackberry and black raspberry both contained cyanidins as primary anthocyanins, yet with contrasting glycosylation profiles. Blackcurrant and maqui berry predominantly contained delphinidins with diglycosylation profile similar to black raspberry. Blueberry contained mono-glycosylated delphinidins, malvidins, and petunidins (opposite of blackberry), while Concord grape was selected for high presence of acylated delphinidins, malvidins, and petunidins (Table 2.2.). Despite diverse anthocyanin profiles, all berries contained sufficient amounts of total anthocyanins to allow for normalized incorporation of berries into HFD. The final diets contained from approximately 0.5% (black raspberry) to 5% (Concord grape) of the freeze-dried whole berry powders (w/w) and delivered 400 µg/g food of total anthocyanins. In this study, animals consumed an average of 2.85 ± 0.34 g food/mouse/day (71.3 mg/kg/day), thus ingesting 1.14 mg/mouse/day (28.5 mg/kg/day) of total anthocyanins. When translated to humans, this treatment was equivalent to consuming 2.4 mg/kg/day or 145 mg/day of total anthocyanins for an average adult (Reagan-Shaw, Nihal, & Ahmad 2008), and could be easily achieved by daily consumption of 1–2 servings of fresh berries (McGhie *et al.* 2003). Normalization for anthocyanin content did not allow us to control the treatments for different sugar levels. All berries had similar carbohydrate profiles (10–17 g/100 g fresh weight

(FW)), however Concord grapes contained more sugars (16 g/100 g FW) than the rest of the berries (4–10 g/100 g FW).

Dietary supplementation with mono-glycosylated cyanidins (blackberry) and di-glycosylated cyanidins (black raspberry) had no effect on body weight, food intake, body composition and metabolic risk factors (fasting blood glucose and insulin sensitivity) in the HFD mice (Figure 2.2, Figure 2.3 and Figure 2.4). Previously, whole powdered black raspberry supplementation did not prevent the development of obesity or improved lipid status in HFD mice (Prior & Wu 2006) and it was suggested that complex glycosylation nature of black raspberry anthocyanins precluded their ability to modulate metabolic health (Prior *et al.* 2010). A similar observation was made for complex acylated cyanidins from purple carrot (Wright *et al.* 2014), di-glycosylated cyanidins from elderberry (Curtis *et al.* 2009), acylated and di-glycosylated cyanidins from blood orange (Giordano *et al.* 2012), and mono-glycosylated cyanidins from jaboticaba (Marques *et al.* 2012). Weak antidiabetic effects were reported in animals for cyanidins from purple corn (Tsuda *et al.* 2003), black soybeans (Kwon *et al.* 2007), purple sweet potato (Hwang *et al.* 2011), mulberry (Wu *et al.* 2013), cherry (Wu *et al.* 2014), chokeberry (Qin & Anderson 2012), or highly purified cyanidin-3-glucoside alone (Guo *et al.* 2012). Taken together, our data strongly suggested that cyanidin-based dietary interventions would be much less effective in alleviating metabolic risk factors than previously thought.

Primary di-glycosylated delphinidins from black currants (non-acylated) and maqui berry showed similar effects on body weight, body composition, and measures of insulin resistance in the HFD animals. While blackcurrant showed higher efficacy at improving the metabolic outcomes associated with obesity and diabetes, supplementation with maqui berry at physiological levels of dietary intake had limited effect on these parameters, likely due to high

proportion of di- and tri-glycosylated anthocyanins in the fruit. Previously, hypoglycemic effects of maqui berry were reported for supraphysiological levels of supplementation with the anthocyanin-enriched extract (100–500 mg/kg) and purified delphinidin 3-sambubioside-5-glucoside (425 mg/kg) (Rojo *et al.* 2012). Beneficial metabolic effects of black currant anthocyanins have been also described (Benn *et al.* 2014; Esposito *et al.* 2015). We observed a similar outcome from consumption of blueberry mono-glycosylated delphinidins, malvidins, and petunidins (non-acylated) as compared to the Concord grape (acylated). Berries with acylated anthocyanin profiles showed less biological activity when consumed in physiological concentrations. Direct comparison between mono-glycosylated anthocyanins from blueberry and di-glycosylated anthocyanins from black currants suggested that supplementation with mono-glycosylated molecules is more beneficial to the metabolic health (Figure 2.2, Figure 2.3 and Figure 2.4). There are two possible explanations for the observed differences between cyanidin- and delphinidin/malvidin/petunidin-type anthocyanins. It is very likely that anthocyanins with the free hydroxyl groups are less stable in the gastrointestinal environment and do not depend on gastrointestinal bacteria for their metabolism and absorption (delphinidin being an exception as it can be *O*-methylated to form petunidin or malvidin in vivo). This property was particularly evident in animals with disrupted gut microbiome (Table 2.3). At the same time, *O*-methylated metabolites from malvidins and petunidins (including *O*-methyl delphinidins) were characterized with increased hydrophobicity at the B-ring of the molecule that reduced the plasma residence time and increased tissue affinity (Ichiyanagi *et al.* 2006), thus being more biologically active. Complex glycosylation and acetylation patterns reduced bioactivity of anthocyanins, but greatly increased their stability in the gastrointestinal tract. When gut microbiome was disrupted with antibiotics, we observed 4–10-fold increase in di-

glycosylated and acylated anthocyanins excreted in feces. The effect was strongest in animals consuming maqui berry; dietary supplementation with 400 µg/g anthocyanins resulted in 339 µg/g anthocyanins excretion rate in feces upon suppression of gastrointestinal bacteria with the antibiotic cocktail (Table 2.3). The data suggested that subjects with gut microbiomes disrupted by a disease or lifestyle modification would have decreased benefits from dietary supplementation with berries as compared to their healthy counterparts, as described for black currants previously (Esposito *et al.* 2015).

The gut microbiota in the LFD mice was dominated by Firmicutes (71%) and Bacteroidetes (15%), and the HDF diet increased this ratio to 89% and 7%, respectively, in strong agreement with previously reported analysis of the mouse gut metagenome (Xiao *et al.* 2015). We observed significant shifts in the gut microbiome profiles towards greater abundance of obligate anaerobes in animals supplemented with different berries, and these effects were most prominent in blackcurrant and blueberry groups (Figure 2.5). Since oxygen removal from the gut depends on food composition, microbial fermentation, and native oxidases secreted into the gut lumen (Johnson & Barbehennb 2000), the changes in the oxygen content of the luminal environment may thereby modulate the composition of the gut microbiota. Indeed, consumption of HFD was associated with increased oxygen tension in all gut compartments, and this effect was reduced by incorporating berries and berry anthocyanins into the diet (Figure 2.6). Conversely, increase in host oxygenation altered luminal oxygenation in the gut and suppressed oxygen-intolerant bacterial populations (Albenberg *et al.* 2014). The presence of marked hypoxia within the lumen of the distal parts of the gastrointestinal tract is therefore consistent with the known abundance of anaerobic bacteria at these sites, and our study indicates that

gastrointestinal oxygenation can be modulated by dietary supplementation with berry anthocyanins.

## 2.5. Conclusions

In conclusion, the data presented in this study agreed with the emerging findings from preclinical and human studies demonstrating that delphinidin- and malvidin-based anthocyanins, at concentrations attainable in human tissues, were more effective at improving key metabolic risk factors than their cyanidin-based counterparts. Complex glycosylation and acetylation patterns reduced bioactivity of anthocyanins, but greatly increased their stability in the gastrointestinal tract. Finally, diets supplemented with berry or anthocyanins reduced gut luminal oxygenation and promoted abundance of obligate anaerobic bacteria from Bacteroidetes and Actinobacteria phyla at these sites. Further work is needed to understand mechanisms that lead to nearly anoxic conditions in the gut lumens, including the relative contributions of host, diet and/or microbial oxidative activity, and their implication to human health.

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## 2.7. Author Contributions

Author contributions were as follows: John Overall, Debora Esposito, Mary Ann Lila, and Slavko Komarnytsky conceived and designed the experiments; John Overall, Arnold Beermann III and Mickey Wilson performed the animal experiments; Sierra A. Bonney

performed microbiome analysis; Mary H. Grace performed HPLC analysis; John Overall and Slavko Komarnytsky analyzed the data; and John Overall, Debora Esposito and Slavko Komarnytsky wrote the paper.

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## CHAPTER 3: CONCLUSIONS & FUTURE DIRECTIONS

Berries and their extracts may afford substantial protection against CMD risk due to their relatively high abundance of a diverse array of therapeutically active phytochemical compounds, especially various polyphenolics such as the ANCs subclass, which represent a particularly promising lead for future investigation and potential for development of nutraceutical interventions or other therapeutic modalities. Whole berries, berry extracts, as well as purified berry polyphenolics and/or berry-derived metabolites may be of interest for development of therapeutic agents to target cardiometabolic risk and ameliorate the burden of obesity-related pathologies such as diabetes and their attendant complications (Burton-freeman *et al.,* 2019). ANC-rich berry-based dietary interventions warrant consideration for use as medical nutrition therapies to manage obesity-related CMD risk factors associated with onset and progression of metabolic syndrome and obesity complications - diabetes, insulin resistance, and chronic inflammation (Basu, 2019). They may represent a low-cost and low-risk lifestyle modification strategy that promotes health by improving physiological resilience generally (Black and Bowman, 2020; Calvano *et al.,* 2019). ANCs and their metabolites may be highly effective in counteracting the insidious low-grade metabolic inflammation and vascular/endothelial dysfunction that has been well established in its connection with risk of diabetes, obesity, and cardiovascular disease (Hameed *et al.* 2020; Mutie *et al.* 2020). Furthermore, they may warrant consideration in treatment of age-related degenerative disorders such as sarcopenic muscle loss, impaired wound healing, and age-related loss of regenerative capacity affecting skin and other connective tissues that detract from normal tissue architecture and impinge upon functionality (Ho *et al.* 2017; Esposito *et al.* 2019; Xiong *et al.* 2019). Notably, recent evidence supports potential suitability of parent ANCs as well as phenolic metabolites that may be found in tissues

and circulation after berry consumption for use as therapeutics against tissue/organ dysfunction or damage associated with pathological aging, such as conditions associated with low muscle mass or deficient lean body mass as in sarcopenia or muscle wasting disorders (Blum *et al.* 2020; Ho *et al.*, 2017; Saclier *et al.* 2020). Further research is warranted to expand the current evidence base and move towards identification of optimal berry-derived therapeutic lead structures for application in regenerative medicine interventions that target sarcopenic risk and facilitate preservation of lean body mass and healthy body composition through influencing the processes controlling regulation of myogenesis and muscle stem cell dynamics, and that may repair healthy tissue architecture and restore normal functionality in aged or damage skin and other connective tissues in the context of aging or injury. Our research suggests ANCs may be effective therapeutic agents to target age-related muscle loss and thereby improve functional limitations associated with sarcopenic frailty (Jia 2019). They may also be targeted towards aging-related impairments in the wound healing response, and aging changes in skin tissues such as fibrosis, fragmentation of ECM collagens, and loss of hydration and elasticity (Esposito 2019).

In our study presented in chapter 2 of this thesis, ANC-standardized berry extracts were incorporated in the obesogenic diets of mice; a large amount (60%) of fat was used to simulate so-called 'Western' high-fat diet – and supplementation of these diets with selected berry treatments produced beneficial, if modest, impacts on HFD-induced obesity and metabolic complications related to body composition, glucose regulation and insulin function. These results were largely statistically insignificant, and thus were not overwhelmingly in favor of berry efficacy in this particular experimental context. Specifically, a tangible beneficial impact on factors relating to obesity, diabetes, and CMD risk in conjunction with obesogenic lifestyle (Western high-fat diet) was associated only with three particular berries – blueberry, concord

grape, and blackcurrant. Berry dietary interventions were however associated with changes in intestinal microbial community composition and ANCs from blackcurrant specifically reduced localized oxygenation in parts of the intestinal lumen, which supports a plethora of evidence from other investigations indicating impacts of berries, and their polyphenolic and/or ANC extracts on regulation of gut microbial species abundance and functionality (Cremonini *et al.* 2019). Moreover, our subsequent analysis of gene expression profiles from banked muscle and visceral fat tissues harvested from mice from the aforementioned experiment revealed significant impacts of berry diets on expression of myogenic regulatory factors (MRFs), which are genetic factors linked to regulation of new muscle fiber growth and postnatal muscle development; thus suggesting that berry interventions, through a myogenic regulatory mechanism, may have the potential to favorably modulate muscle development and thereby protect against muscle loss in sarcopenia and buffer against age-related changes in body composition (Xiong *et al.* 2019).

In future, comprehensive preclinical and *in vitro* mechanistic research investigations are highly necessary to elucidate the cellular and molecular underpinnings of their therapeutic actions. Additional human feeding trials are also exceedingly crucial to conclusively demonstrate the effectiveness of ANC-based dietary approaches in health promotion and chronic disease prevention (Daneshzad *et al.* 2019). Critically, whether and to what extent they may exert clinically relevant impacts on the course of obesity, diabetes, and cardiometabolic risk factors, and on pathological aging disorders such as sarcopenic frailty or impaired wound healing; it is important to evaluate in the most relevant clinical populations such as patients who are aging, obese, and/or sarcopenic (*i.e.* frail), and especially in populations known to exhibit multiple comorbidities in combination that are considered to be at particularly elevated risk for CMDs and pathological aging complications. Although the health effects mediated by ANC intake may vary

based on the structural characteristics of individual ANCs, or on their specific composition as represented in a particular berry source or food matrix, one thing is clear – further research is crucial to elaborate their therapeutic significance both *in vivo* and in clinical use; to understand the true extent of their divergent dispositions after oral intake associated with divergent proclivities to host metabolism, especially by actions of gut microbiota leading to heterogenous circulating metabolites; and finally to elucidate the molecular mechanisms by which different ANCs and their metabolites exert these distinct effects *in vivo.* The broad diversity of the polyphenolic arsenal present in various berry species is expanded even further upon consideration of the vast array of derived metabolites – including ubiquitous breakdown products common to many different polyphenolics such as hippuric acid, as well as more unique metabolite structures – many in this latter category have yet to be explored in scientific investigation, and it has been proposed that a great many yet remain even to be characterized (Kay *et al.*, 2020). Thus, berry polyphenolic extracts, their constituents, derivative metabolites, and catabolic byproducts collectively represent a rich botanical reservoir of therapeutic molecules which may be ideally suited to a range of therapeutic indications. Not only are parent structures important to take into account when defining bioavailability and bioactivity of phytochemicals after berry consumption; it is equally crucial, if not even more so, to account for their structural metabolites and catabolic end products resulting from host metabolic processes or by catalytic actions of gut microbiota. Ideally, future explorations should aim at clarifying the individual therapeutic activities of berry ANCs and other polyphenolics when consumed in isolation (as in extracts that have been highly purified) as well as the relative contribution of individual subclasses, and specific compounds and their metabolites contained therein, to the collective profile of therapeutic activity associated with the intake of whole berries or of extracts.

If possible, systematic examination of the bioavailability and bioactivity patterns associated with each subclass of polyphenolic constituents, taking into account their respective breakdown products following host metabolism and gut microbial metabolism, would be of immense scientific and biomedical value (Kawabata, Yoshioka & Terao, 2019). However, it must be said that despite the tremendous potential rewards of such an undertaking, this would represent a truly Herculean effort given currently available methodological and technological limitations (Chandra *et al.,* 2019). The advent of metabolomics and other omics tools represents an important step towards this goal, however, much work is still needed in future before such an idea could be considered tenable.

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# TABLES

Table 2. 1. **Anthocyanin profiles of whole freeze-dried berry powders.** Total anthocyanins were determined by the pH differential (Lee *et al.* 2005), while individual peaks were quantified by HPLC (Zhang *et al.* 2012).

| ***Berry*** | ***Total Anthocyanins (mg/g Dry Weight)*** | ***Individual Anthocyanins*** | ***Percent % (Total Anthocyanins)*** |
|---|---|---|---|
| Blackberry (BB) | 9.42 ± 0.03 | 1. Cyanidin-3-*O*-glucoside | 77.58 |
| | | 2. Cyanidin-3-*O*-rutinoside | 6.9 |
| | | 3. Non-identified | *trace* |
| | | 4. Cyanidin-3-*O*-(malonyl)glucoside | 15.52 |
| | | 5. Cyanidin-3-*O*-(dioxalyl)glucoside | *trace* |
| | | | |
| Black currant (BC) | 16.42 ± 0.24 | 1. Delphinidin-3-*O*-glucoside | 14.61 |
| | | 2. Delphinidin-3-*O*-rutinoside | 43.92 |
| | | 3. Cyanidin-3-*O*-rutinoside | 39.54 |
| | | 4. Cyanidin-3-*O*-glucoside | 2.01 |
| | | | |
| Black raspberry (BR) | 24.75 ± 1.19 | 1. Cyanidin-3-*O*-sambubioside | 8.61 |
| | | 2. Cyanidin-3-*O*-glucoside | 8.62 |
| | | 3. Cyanidin-3-*O*-(xylosyl)rutinoside | 73.62 |
| | | 4. Cyanidin-3-*O*-rutinoside | 8.18 |
| | | 5. Pelargonidin-3-*O*-rutinoside | 1.13 |
| | | | |
| Blueberry (BL) | 9.33 ± 0.26 | 1. Delphinidin-3-*O*-galactoside | 9.21 |
| | | 2. Delphinidin-3-*O*-glucoside | 10.22 |
| | | 3. Cyanidin-3-*O*-galactoside | 4.12 |
| | | 4. Delphinidin-3-*O*-arabinoside | 6.03 |
| | | 5. Cyanidin-3-*O*-glucoside | 4.34 |
| | | 6. Petunidin-3-*O*-galactoside | 7.72 |
| | | 7. Cyanidin-3-*O*-arabinoside | 2.63 |
| | | 8. Petunidin-3-*O*-glucoside | 7.51 |
| | | 9. Peonidin-3-*O*-galactoside | 1.44 |
| | | 10. Petunidin-3-*O*-arabinoside | 5.33 |
| | | 11. Malvidin-3-*O*-galactoside | 8.81 |
| | | 12. Malvidin-3-*O*-glucoside | 12.7 |
| | | 13. Malvidin-3-*O*-arabinoside | 5.12 |
| | | 14. Delphinidin-3-*O*-(acetyl)glucoside | 3.54 |

Table 2. 1. (Continued)

| | | | |
|---|---|---|---|
| Blueberry (BL) | | 15. Cyanidin-3-*O*-(acetyl)glucoside | 1.43 |
| | | 16. Malvidin-3-*O*-(acetyl)galactoside | 2.11 |
| | | 17. Petunidin-3-*O*-(acetyl)glucoside | 2.24 |
| | | 18. Malvidin-3-*O*-(acetyl)glucoside | 4.83 |
| | | | |
| Concord grape (CG) | 2.37 ± 0.03 | 1. Delphinidin-3-*O*-glucoside | 13.61 |
| | | 2. Cyanidin-3-*O*-glucoside | 12.01 |
| | | 3. Petunidin-3-*O*-glucoside | 7.41 |
| | | 4. Peonidin-3-*O*-glucoside | 5.92 |
| | | 5. Malvidin-3-*O*-glucoside | 6.42 |
| | | 6. Delphinidin-3-*O*-(acetyl)glucoside | 5.75 |
| | | 7. Delphinidin-3,5-*O*-(coumaroyl)diglucoside | 5.6 |
| | | 8. Cyanidin-3-*O*-(acetyl)glucoside | 5.31 |
| | | 9. Cyanidin-3,5-*O*-(coumaroyl)diglucoside | 6.55 |
| | | 10. Petunidin-3-*O*-(acetyl)glucoside | *trace* |
| | | 11. Malvidin-3,5-*O*-(coumaroyl)diglucoside | *trace* |
| | | 12. Peonidin-3,5-*O*-(coumaroyl)diglucoside | *trace* |
| | | 13. Peonidin-3-*O*-(acetyl)glucoside | *Trace* |
| | | 14. Delphinidin-3,5-*O*-(coumaroyl)diglucoside | 11.36 |
| | | 15. Cyanidin-3-*O*-(coumaroyl)glucoside | 8.05 |
| | | 16. Petunidin-3-*O*-(coumaroyl)glucoside | 6.24 |
| | | 17. Peonidin-3-*O*-(coumaroyl)glucoside | 5.77 |
| | | 18. Malvidin-3-*O*-(coumaroyl)glucoside | *trace* |
| | | | |
| Maqui berry (MB) | 10.95 ± 0.12 | 1. Delphinidin-3-*O*-sambubioside-5-*O*-glucoside | 15.36 |
| | | 2. Delphinidin-3,5-*O*-diglucoside | 23.91 |
| | | 3. Cyanidin-3,5-*O*-diglucoside | *trace* |
| | | 4. Cyanidin-3-*O*-sambubioside-5-*O*-glucoside | 11.32 |
| | | 5. Delphinidin-3-*O*-sambubioside | 7.76 |
| | | 6. Delphinidin-3-*O*-glucoside | 31.26 |
| | | 7. Cyanidin-3-*O*-glucoside | 6.86 |
| | | 8. Cyanidin-3-*O*-sambubioside | 3.53 |

Table 2. 2. **Summary of anthocyanin structural diversity in whole freeze-dried berry powders.** Represented in proportion to total as percent % of total representation according to: (A) Anthocyanidin type, (B) Glycosylation, and (C) Acetylation.

| **Berry Treatment** | **(A) Anthocyanidin (Aglycone) as a percent % of Total Anthocyanidins** | | | | | **(B) Glycosylation Ratio (%Di / %Mono)** | **(C) Acetylation Ratio (%Acetyl / %Non-)** |
|---|---|---|---|---|---|---|---|
| | **Cy** | **De** | **Mv** | **Pt** | **Peo** | | |
| Blackberry (BB) | 100 | 0 | 0 | 0 | 0 | 7/93 | 16/84 |
| Black currant (BC) | 42 | 58 | 0 | 0 | 0 | 84/16 | 0/100 |
| Black raspberry (BR) | 99 | 0 | 0 | 0 | 0 | 91/9 | 0/100 |
| Blueberry (BL) | 12 | 29 | 34 | 23 | 2 | 0/100 | 13/87 |
| Concord grape (CG) | 32 | 37 | 7 | 19 | 7 | 23/77 | 54/46 |
| Maqui berry (MB) | 21 | 78 | 0 | 0 | 0 | 62/38 | 0/100 |

Table 2. 3. **Total Anthocyanins recovered from feces as µg/g dry weight (DW).** Berry diets were normalized to contain 400 µg total anthocyanins / g of food weight – thus anthocyanins represented 0.04% of the total food by weight. This was achieved by incorporating 0.5%–5% of whole freeze-dried berries in each diet to account for the slight variability in anthocyanin proportions within the individual berry concentrates used to prepare each diet; n.d., not detected.

| **Diet** | **Time on Diet, Weeks** ----- *(µg/g DW ACNs detected in feces)* | | | |
|---|---|---|---|---|
| | **4** | **8** | **9 (1 Week Post-Antibiotic)** | **12** |
| HFD | *n.d.* | *n.d.* | *n.d.* | *n.d.* |
| BB | *2.37 ± 0.08* | *3.48 ± 0.10* | *8.51 ± 0.18* | *4.83 ± 1.13* |
| BC | *26.35 ± 1.25* | *34.26 ± 0.14* | *326.82 ± 31.89* | *48.17 ± 2.74* |
| BR | *44.93 ± 6.64* | *62.10 ± 9.42* | *120.97 ± 2.13* | *83.93 ± 1.30* |
| BL | *5.30 ± 0.66* | *17.05 ± 3.10* | *46.57 ± 6.00* | *18.57 ± 0.91* |
| CG | *9.24 ± 5.13* | *22.41 ± 3.74* | *260.58 ± 10.46* | *34.26 ± 0.60* |
| MB | *30.38 ± 1.18* | *31.22 ± 1.37* | *339.32 ± 52.69* | *172.16 ± 5.44* |

## FIGURES

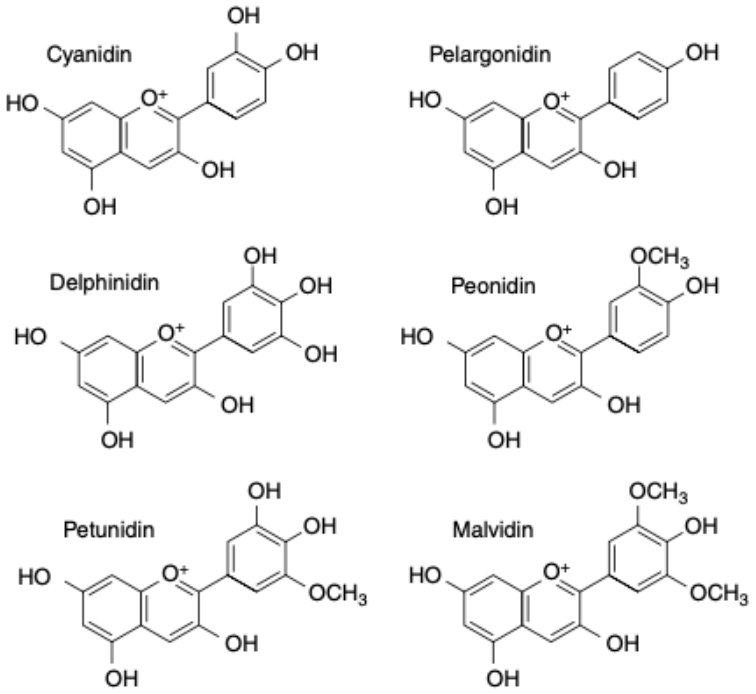


Figure 1. 1. **Structural variation of the most common naturally occurring anthocyanin aglycones (anthocyanidins)**.

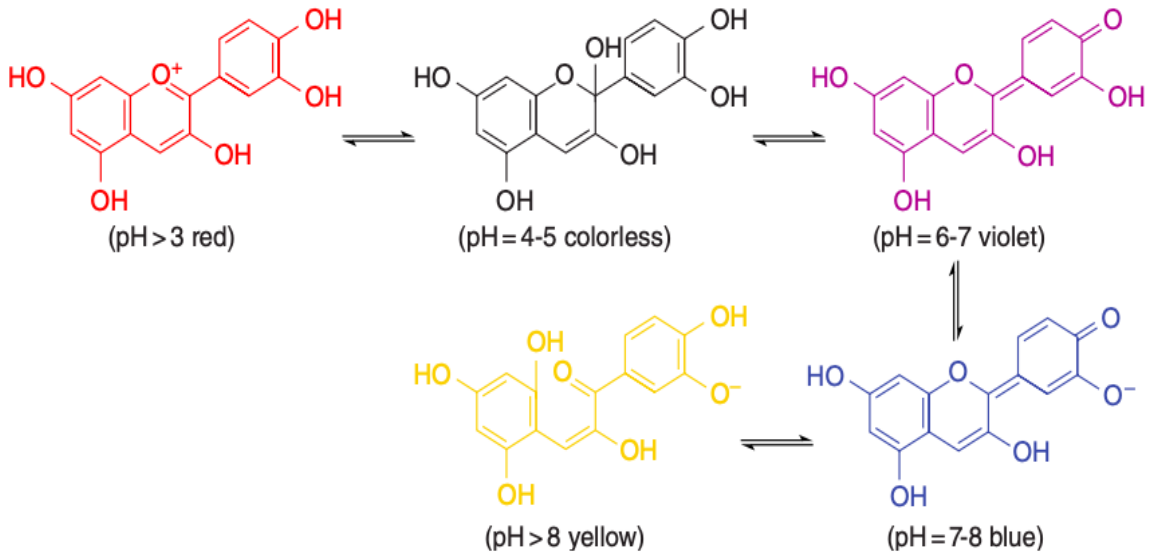


Figure 1. 2. **Effect of pH on chemical structure and coloration of anthocyanins**.

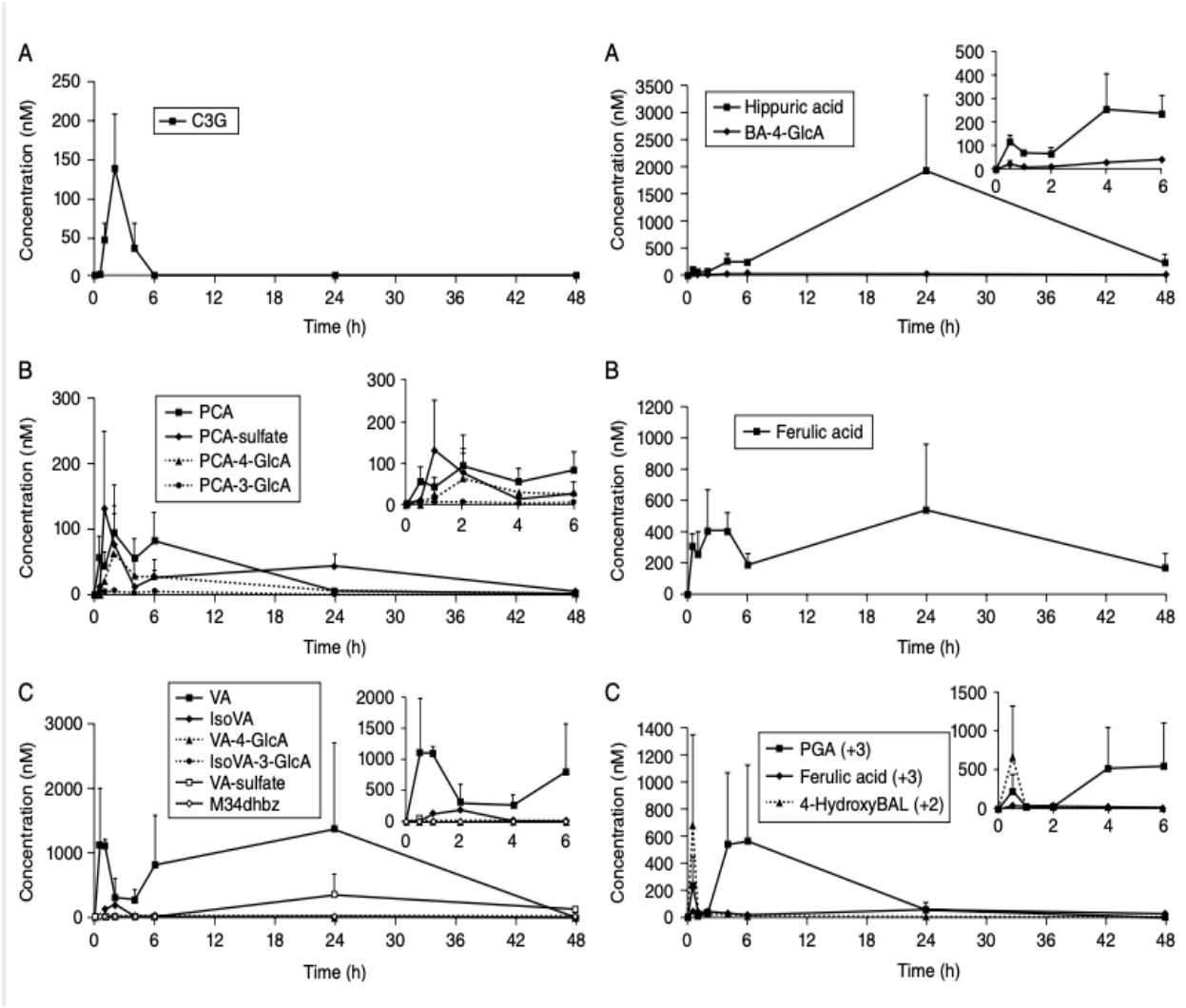


Figure 1. 3. **Pharmacokinetics of cyanidin-3-glucoside (left panel, A), its major breakdown metabolites (left panel, B and C) and phenolic acids (right panel, A–C) in humans**. Originally reproduced from de Ferrars *et al.* (2014); subsequently adapted from Overall, Komarnytsky, and Lila (2017).

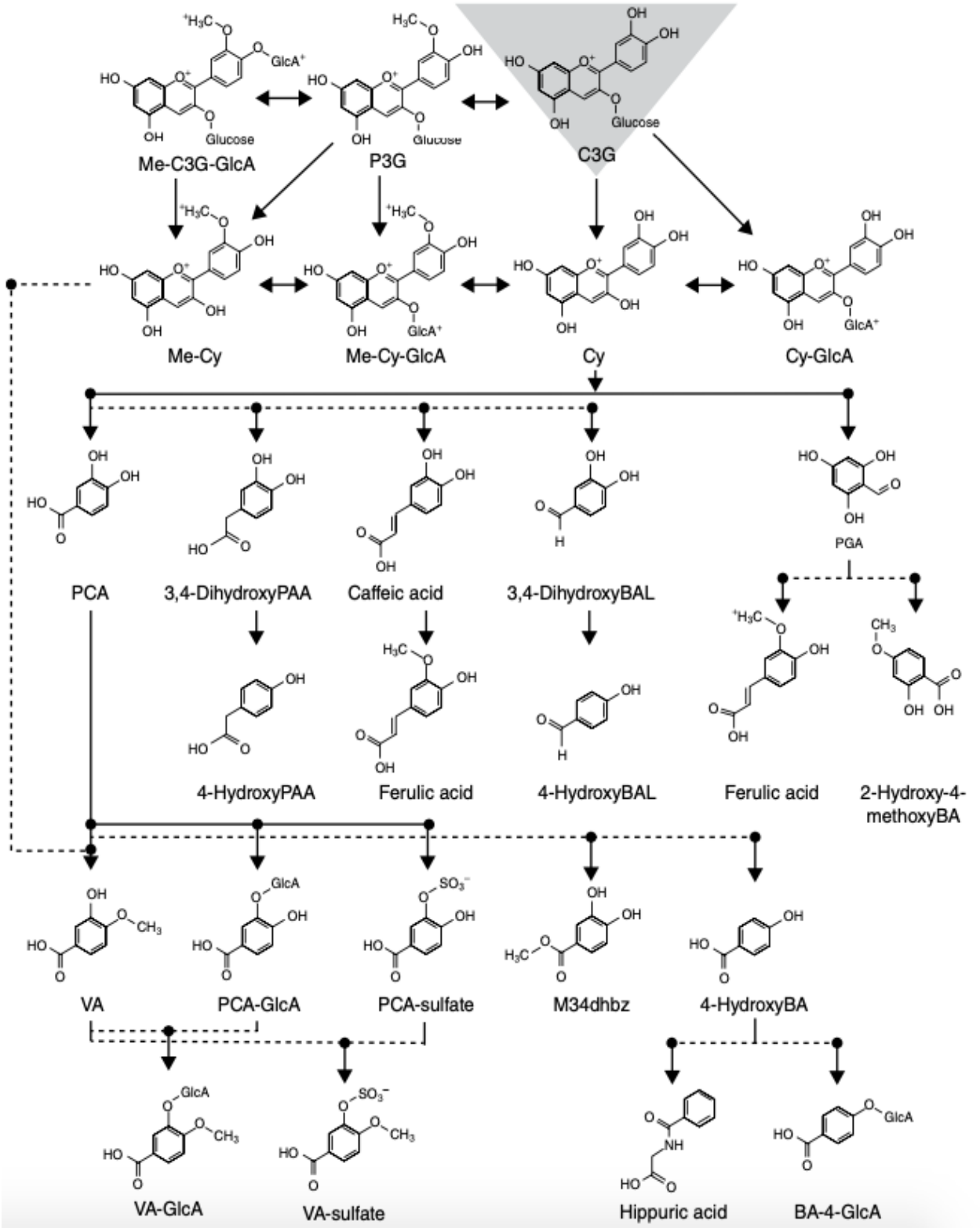


Figure 1. 4. **Proposed pathway for the metabolism of cyanidin-3-glucoside in humans**. Originally reproduced from de Ferrars *et al.* (2014); subsequently adapted from Overall, Komarnytsky, & Lila (2017).

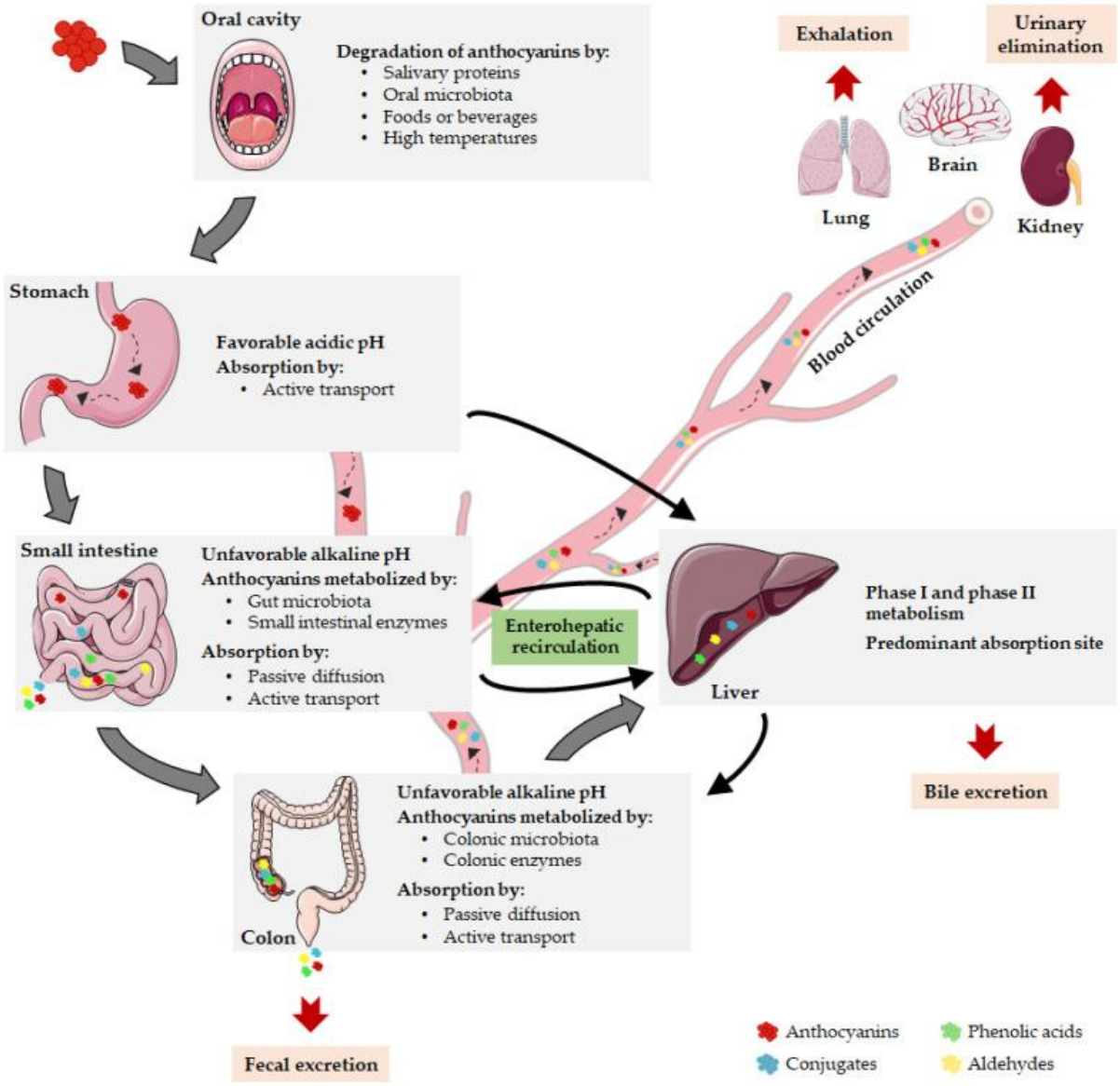


Figure 1. 5. **Proposed route of absorption, uptake and distribution of ANCs after *in vivo* consumption.** Reproduced from Henriques *et al.* (2020).

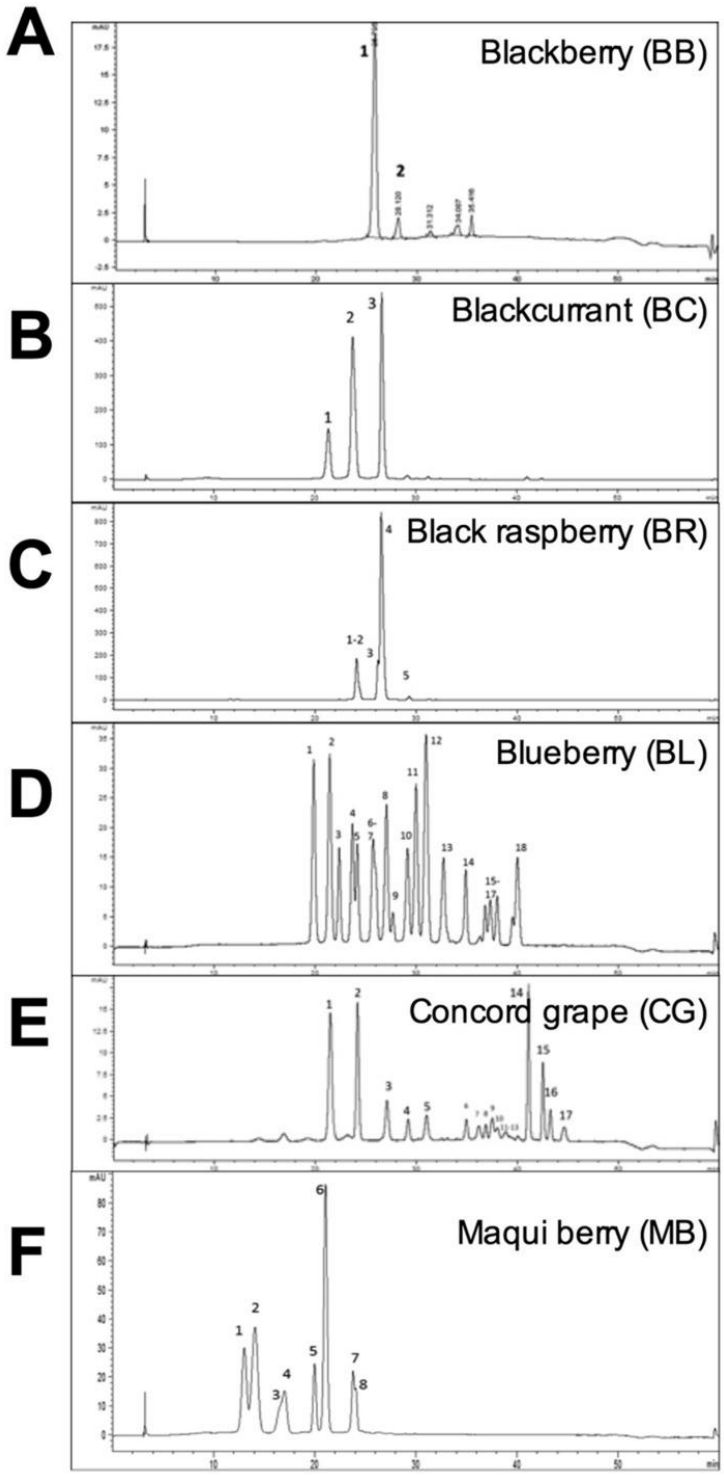


Figure 2. 1. **HPLC analysis of anthocyanins from whole freeze-dried berries.** (A) blackberry, BB ; (B) blackcurrant, BC; (C) black raspberry, BR; (D) blueberry, BL; (E) Concord grape, CG; and (F) maqui berry, MB. Peak numbers correspond with labels in Table 2.1 and present in the same sequence.

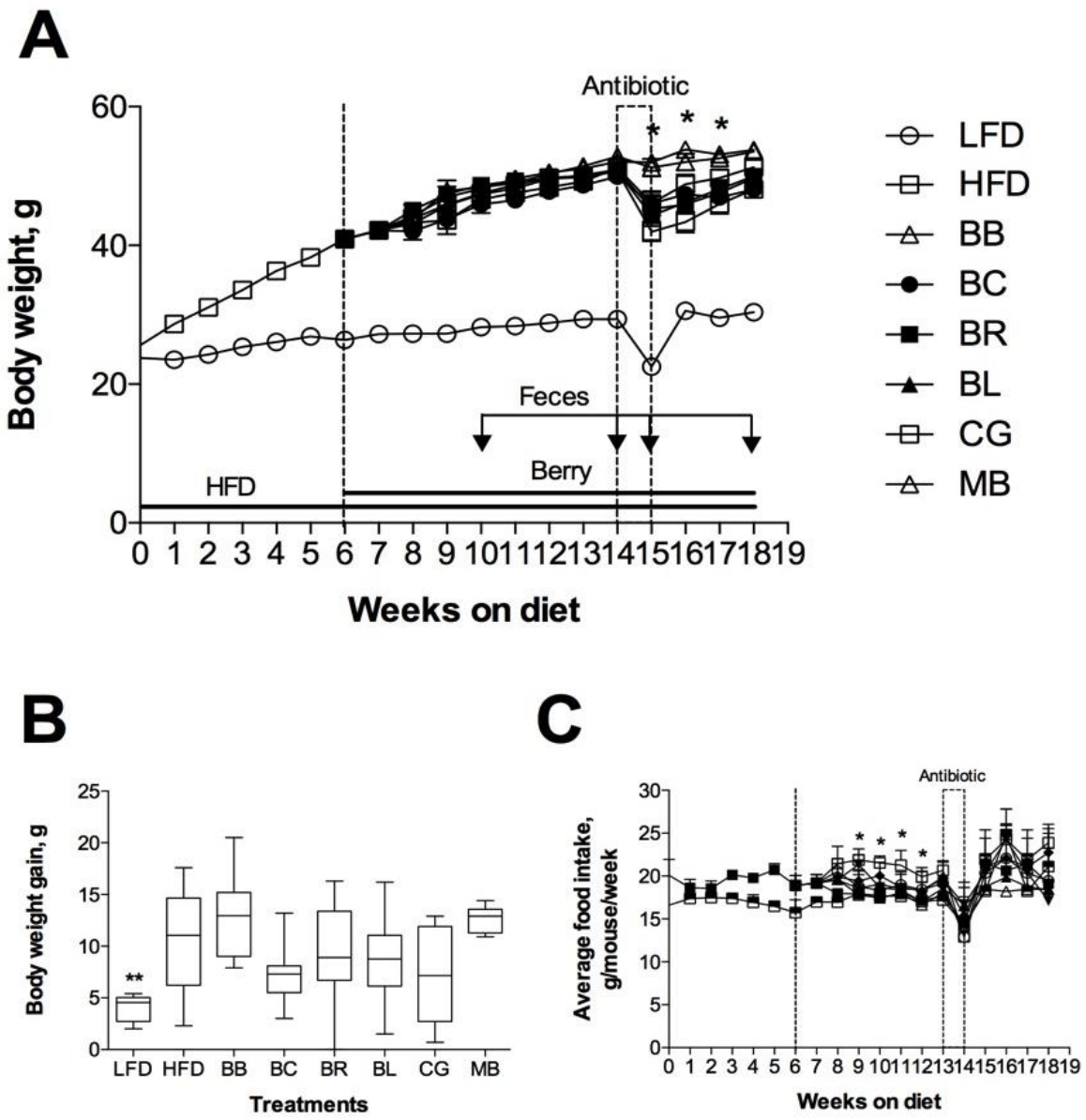


Figure 2. 2. **Effects of berry supplementation on body weight (A); body weight gain (B); and food intake (C) of C57BL/6J mice.** Mice were fed low fat diet (LFD) or high fat (HFD) for six weeks. DIO mice were further randomized to control (HFD) and berry diets (BB, blackberry; BC, blackcurrant; BR, black raspberry; BL, blueberry; CG, Concord grape; MB, maqui berry) normalized to contain 400 µg/g anthocyanins for additional 12 weeks. An antibiotic cocktail was administered for one week in drinking water (Weeks 14–15 of the study) and feces were collected as indicated by arrows. Results are expressed as means ± SEM, $n = 8$. Body weight and food intake were analyzed by 2-factor repeated measures ANOVA, with time and treatment as independent variables. * $p < 0.05$, ** $p < 0.01$ vs. HFD control.

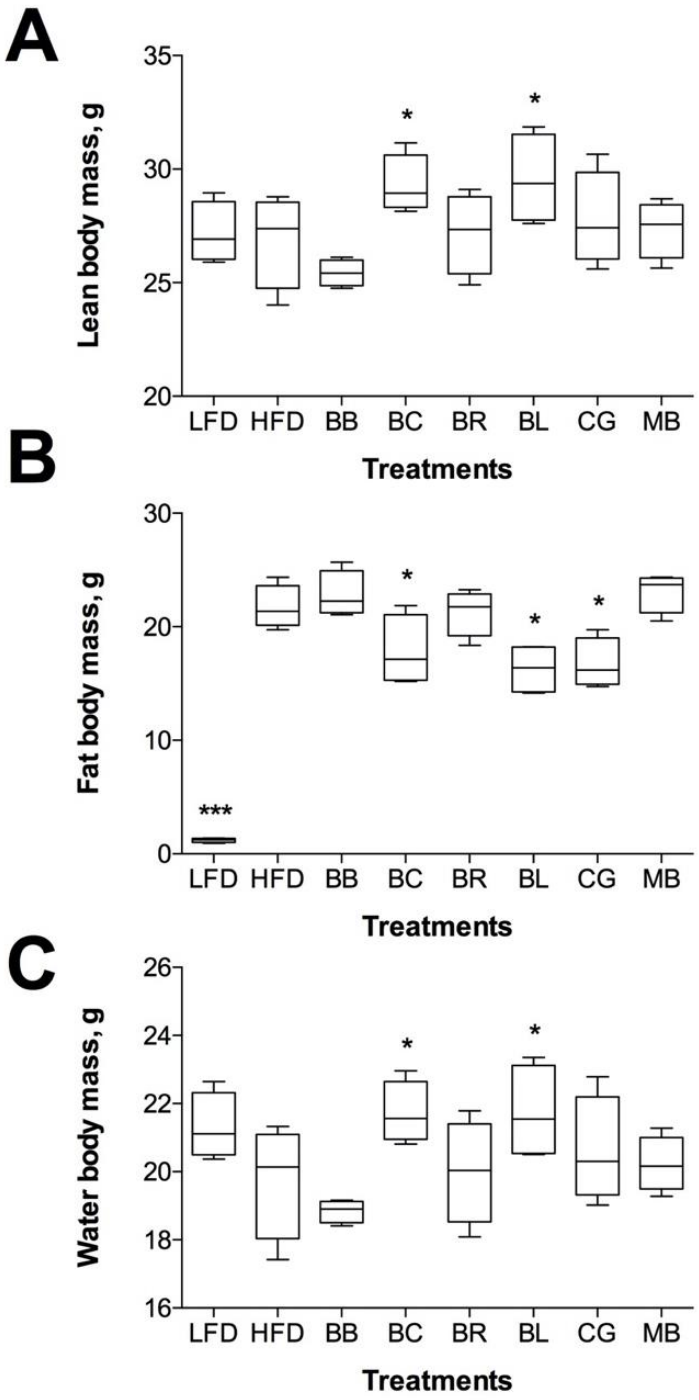


Figure 2. 3. **Effects of berry supplementation on body composition**. (A) lean body mass; (B) fat body mass; and (C) total water. Data reported as means ± SEM. * $p < 0.05$ and *** $p < 0.001$ when compared to HFD by one-way ANOVA followed by Dunnett's post hoc test.

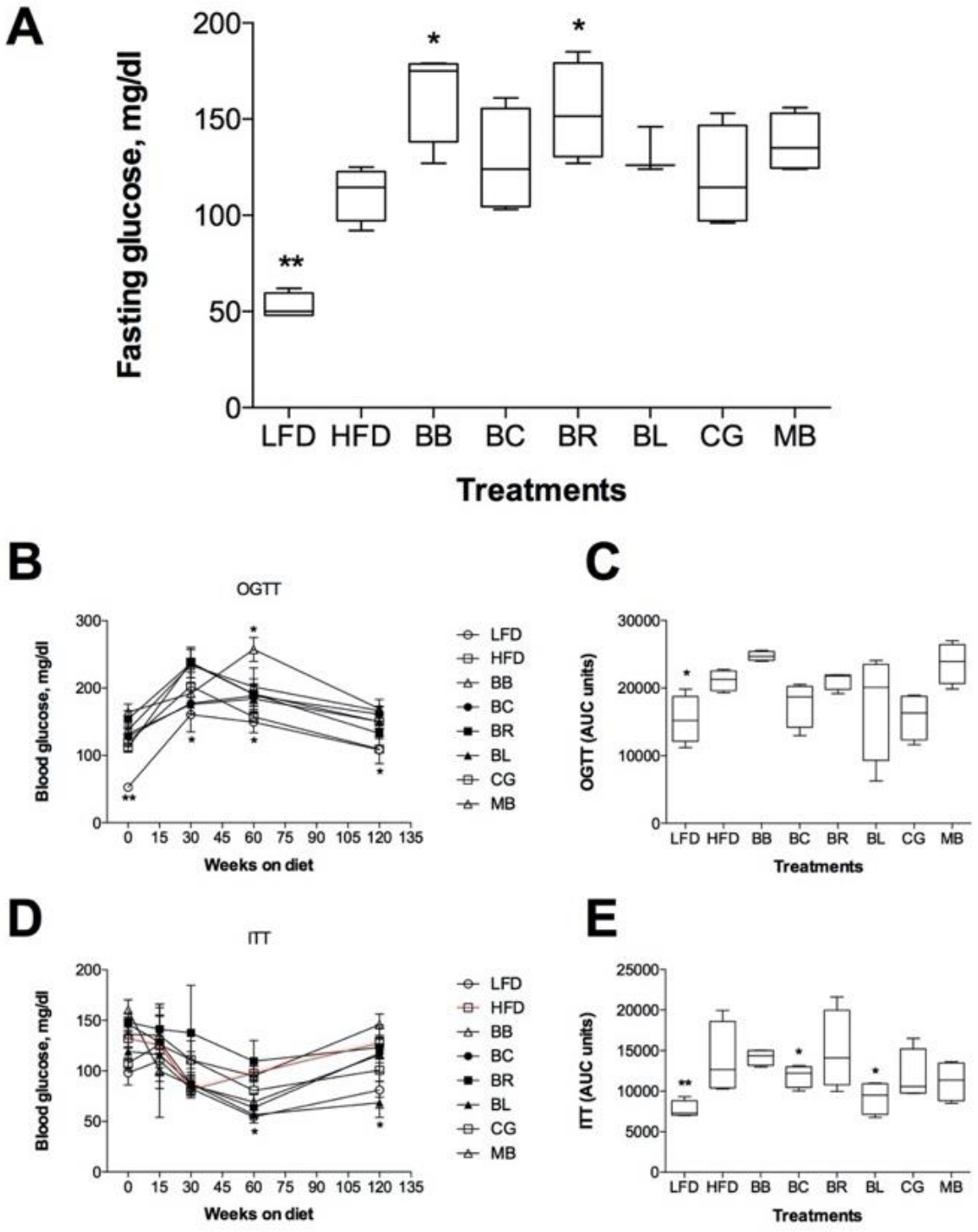


Figure 2. 4. **Insulin-sensitizing effect of berry supplementation**. (A) Fasting blood glucose; (B) oral glucose tolerance test; and (C) respective AUCs; and (D) insulin tolerance test; and (E) respective AUCs. Data are reported as means ± SEM. * $p < 0.05$ and ** $p < 0.01$ when compared by two-factor repeated measures ANOVA (B,D); or one-way ANOVA followed by Dunnett's post hoc test.

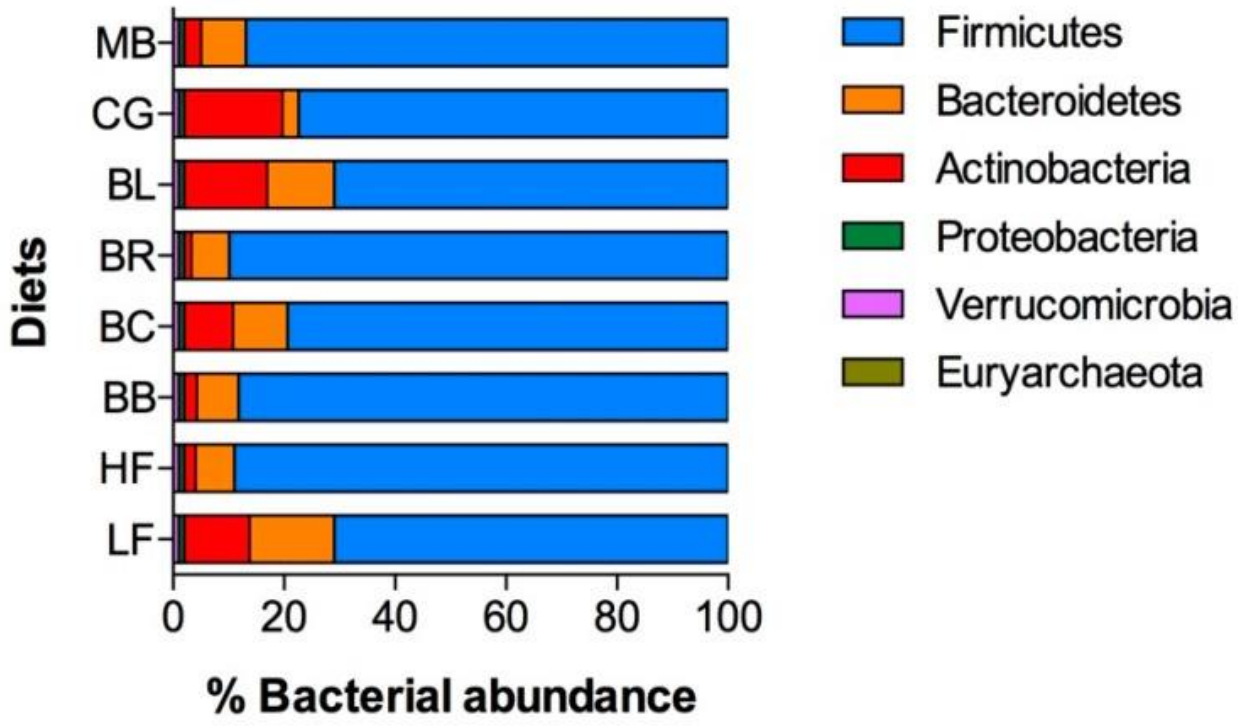


Figure 2. 5. **Changes in gut microbiota profiles following 12-week berry supplementation.** Relative abundance of bacteria phyla in feces samples as measured by GULDA qPCR array indicated significant increase in obligate anaerobe populations of Bacteroidetes and Actinobacteria in berry-supplemented HFD animals (especially in blueberry and blackcurrant), similar to the LFD controls.

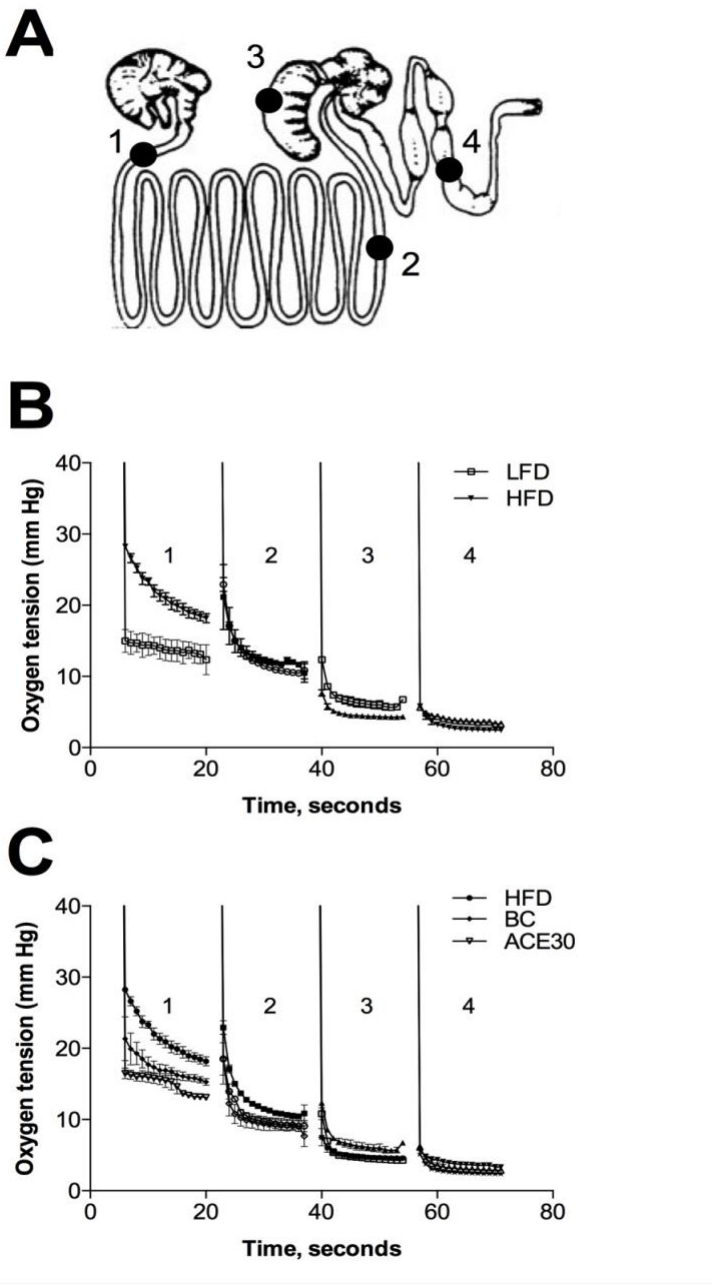


Figure 2. 6. **Gut luminal oxygenation profiles of different gastrointestinal regions of mice following 12-week berry supplementation**. (A) schematic representation of mouse gastrointestinal tract including duodenum (1), ileum (2), cecum (3), and colon (4) sections; (B) oxygen tension curves of LFD and HFD animals indicated significant increases in gut luminal oxygen content of the HFD animals; and (C) oxygen tension curves in mice supplemented with blackcurrant or blackcurrant anthocyanins (ACE30 extract) showed decreased oxygen concentration within the gastrointestinal lumen when compared to HFD controls. Data are reported as means ± SEM. * $p < 0.05$ when compared to by two-factor repeated measures ANOVA.