Effects of Anthocyanins on the Immune and Cardiovascular System

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Anthocyanins are increasingly being recognized for their potential health benefits, including effects on the immune and cardiovascular system. This PhD research project addressed some key research gaps by evaluating the effects of anthocyanins and an anthocyanin-rich blackcurrant beverage on markers of immunity and cardiovascular disease (CVD) risk.

Cellular screening models examined the anti-inflammatory effects of pelargonidin-3-O-glucoside and three of its plasma metabolites in THP-1 monocytes, THP-1 macrophages and whole blood cultures. There were modest effects on the production of interleukin (IL)-6 and IL-10, while no effects were observed on IL-1β, IL-8 and tumor necrosis factor-α. The effects were dependent on the experimental model employed and the data suggest differential anti-inflammatory potencies of the tested phenolic compounds and only modest effect sizes.

The whole blood culture model also examined, for the first time, the effects of pelargonidin-3-O-glucoside and three of its plasma metabolites on phagocytosis and oxidative burst activity, but there were no significant effects. Subsequent studies should explore other immunomodulatory effects of dietary anthocyanins.

An acute RCT investigated, for the first time, the effects of an anthocyanin-rich blackcurrant beverage in response to a high-fat meal on selected markers of CVD risk in healthy middle-aged men and women. The anthocyanin-rich blackcurrant beverage significantly improved flow-mediated dilation, platelet function, systolic blood pressure and IL-8 concentrations. The effects were observed with 711 mg anthocyanins, which corresponds to 120 mg fresh blackcurrants, an amount that is high but achievable through the diet. There were no significant effects of the beverage on diastolic blood pressure, arterial stiffness, plasma concentrations of nitrite, nitrate, or numbers of endothelial or platelet microparticles. In conclusion, the data suggest that anthocyanins can attenuate deleterious effects of a dietary fat challenge and could represent an effective tool for CVD prevention.

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Contents

List of publications

Published paper

Amini A M, M usz K , S pencer J PE, Y aqoob P . 2017. Pelargonidin-3-O-glucoside and i ts metabolites have modest anti-inflammatory effects in human whole blood cultures. *Nutrition Research* 46, 88-95 (**Appendix 1**, the published paper is based on **Chapter 3** of this thesis).

Published abstract

Amini AM, Rodriguez-Mateos A, Spencer JPE, Yaqoob P. 2013. Stability of anthocyanins *in vitro*. *Proceedings of the Nutrition Society* 72 (OCE4): E198 (**Appendix 2**).

Published book chapter

Corona G, Vazour D, **Amini A**, Spencer JPE. The impact of gastrointestinal modifications, blood-brain barrier transport, and intracellular metabolism on pol yphenol bi oavailability: an overview. In: Watson RR, Preedy VR, Zibadi S, ed. Polyphenols in human health and disease. London: Elsevier, 2013: 591-604 (**Appendix 3**).

Manuscript submitted for publication

Amini A M, S pencer J PE, Y aqoob P . Effects o f a s trawberry-derived anthocyanin a nd i ts metabolites on l ipopolysaccharide-stimulated c ytokine production b y THP-1 m onocytes and macrophages (the submitted manuscript is based on **Chapter 2** of this thesis and it is currently under review for publication in *Cytokine*).

Manuscript prepared for submission

Amini AM, Austermann K, Králová D, Serra G, Ibrahim IS, Zhou R, Corona G, Sagi-Kiss V, Bergillos-Meca T , Aboufarrag H , K roon P , S pencer J PE, Y aqoob P . A cute e ffects of an anthocyanin-rich b lackcurrant b everage o n markers o f c ardiovascular d isease r isk in h ealthy volunteers: a randomized, double-blind, placebo-controlled, crossover trial (**Chapter 4** of this thesis, awaiting d ata o n polyphenol analysis of b lood and u rine s amples, submission to *The American Journal of Clinical Nutrition* due).

List of abbreviations

4-HBA	4-hydroxybenzoic acid
ACN	anthocyanin
ADP	adenosine diphosphate
BHF	British Heart Foundation
BMI	body mass index
BP	blood pressure
CD40L	CD40 ligand
CHD	coronary heart disease
C_{max}	maximum concentration
CRP	c-reactive protein
CVD	cardiovascular disease
Cy	cyanidin
Cy-3-glc	cyanidin-3-O-glucoside
DBP	diastolic blood pressure
Dp	delphinidin
DVP	digital volume pulse
DVP-RI	digital volume pulse reflection index
DVP-SI	digital volume pulse stiffness index
EFSA	European Food Safety Authority
EMP	endothelial-derived microparticle
ENA-78	epithelial neutrophil-activating protein 78
FAO	Food and Agriculture Organization
FCS	foetal calf serum
FMD	flow-mediated dilation
IL	interleukin
LDI	laser Doppler imaging
LDL	low density lipoprotein
LPS	lipopolysaccharide
MFI	mean fluorescence intensity
MIP-1α	macrophage inflammatory protein 1α
MP	microparticle

Mv	malvidin
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NO	nitric oxide
PCA	protocatechuic acid
PDGF	platelet-derived growth factor
PF-4	platelet factor 4
Pg	pelargonidin
Pg-3-glc	pelargonidin-3-O-glucoside
PGA	phloroglucinaldehyde
PMA	phorbol 12-myristate 13-acetate
PMA	phorbol 12-myristate 13-acetate
PMP	platelet-derived microparticle
Pn	peonidin
PRP	platelet rich plasma
Pt	petunidin
PWA	pulse wave analysis
PWV	pulse wave velocity
RANTES	regulated on activation, normal T-cell expressed and secreted
RCT	randomized controlled trial
SBP	systolic blood pressure
T_{max}	time of maximum concentration
TNF-α	tumor necrosis factor-α
TP	total polyphenols
WHO	World Health Organization

Chapter 1 Introduction and literature review

1.1 Cardiovascular disease (CVD)

CVD is a major cause of morbidity and mortality throughout the world. In 2012, nearly a third of all global deaths (56 million) were attributed to CVD (17.5 million). Of these, the majority (7.4 million) were caused by coronary heart disease (CHD, also known as ischemic heart disease), followed by 6.7 million stroke deaths (World Health Organization [WHO] 2014). Therefore, there is continued need and importance to develop strategies that reduce the CVD burden. Whilst there are some non-modifiable CVD risk factors, a great proportion of CVDs are preventable through lifestyle changes. The following section will provide an introduction to atherosclerosis, the underlying cause of most CVDs, and discuss the modifiable and non-modifiable risk factors for CVD.

1.1.1 Atherosclerosis

Atherosclerosis is a condition that develops over several decades. It is characterized by a chronic inflammation of the arterial walls of large or medium-sized arteries, causing changes in their morphology and structure. The disease progression can be subdivided into four stages (Figure 1) (Ross 1999).

- Stage 1: Damage to the vascular endothelium is considered to be the initiating step and can be caused by several factors, for example increased plasma low density lipoprotein (LDL) cholesterol concentrations, smoking-derived toxins and hypertension. The resulting endothelial dysfunction is characterized by increased endothelial permeability as well as attachment and migration of monocytes into the subendothelial space (Figure 1, stage 1).
- Stage 2: The monocytes differentiate to macrophages, engulf and oxidize LDL cholesterol and turn into foam cells. Over time, the foam cells aggregate and form visible fatty streaks,

which continuously increase in size through continued recruitment of monocytes and accumulation of LDL cholesterol containing macrophages. The loss in the protective function of the endothelium also facilitates platelet recruitment and activation (Figure 1, stage 2).

- Stage 3: As the lesion increases in size, it gradually occludes the blood vessel, causing a reduction in blood flow. The foam cells release inflammatory molecules and growth factors causing smooth muscle cell migration into the fatty plaque and the formation of an intermediate lesion. Continued inflammation leads to further accumulation of macrophages and a large atherosclerotic lesion with a core of dead cells and a fibrous cap (termed atheroma) can develop (Figure 1, stage 3).
- Stage 4: The fibrous cap can rupture causing a thrombus that can result in a heart attack or a thrombotic stroke if the vessel lumen is completely obstructed (Figure 1, stage 4) (Ross 1999).

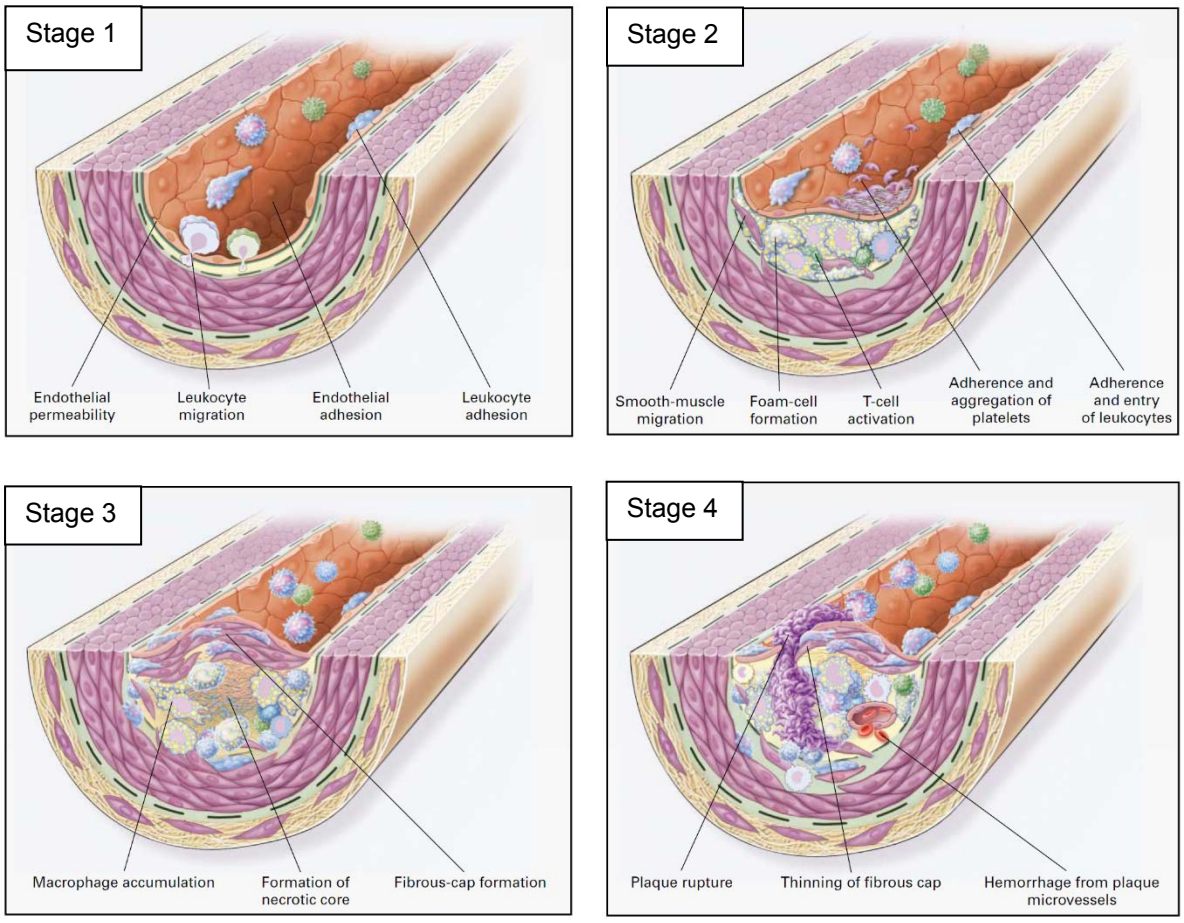


Figure 1 Development of atherosclerosis (Ross 1999).

1.1.2 CVD risk markers

CVD has a multifactorial etiology and a number of risk factors have been associated with increased likelihood of developing CVD which are described in the following section.

1.1.2.1 Non-modifiable CVD risk factors

Age is a major non-modifiable risk factor for CVD. The CVD incidence increases with age and the majority of fatal CVD events occur in those older than 75 years of age. While gender does not affect the absolute risk of CVD mortality (78,240 men and 76,399 women died of CVD in the United Kingdom in 2014), men have a greater risk of CVD mortality at an age of under 75 years than women (British Heart Foundation [BHF] 2015). In addition, there is also evidence that the development of CVD involves a genetic component (Kullo and Ding 2007, Lloyd-Jones 2004) and that ethnicity and socioeconomic factors play a role (BHF 2015).

1.1.2.2 Modifiable CVD risk factors

Evidence suggests that environmental and behavioral factors greatly contribute to the development of CVD. This is supported by data from migration studies, it has for example been demonstrated that CHD rates increase if people move from a low-risk country (such as Japan) to a high-risk country (such as the USA) (Mann 2010). Indeed, a number of well-established modifiable factors have been associated with increased risk of CVD and these include

- smoking (Teo 2006, Hardoon 2008, Erhardt 2009, Ockene and Miller 1997),
- hypercholesteremia (Stamler 1986, Jackson 2012, Lewington 2007),
- hypertension (Lim 2012, Stamler 1986, Macmahon 1990),
- diabetes mellitus (Mazzone 2008, Fox 2007),
- overweight/obesity (Guh 2009) and
- physical inactivity (Thompson 2003).

1.1.2.3 Dietary factors

In addition to the above, dietary factors have been shown to play an important role in the etiology and management of CVD. The WHO/Food and Agriculture Organization (FAO) report on diet, nutrition and prevention of chronic diseases (2003) ruled that there is convincing evidence for a cardioprotective effect of fish and fish oils, fruit and vegetables, linoleic acid, potassium and low to moderate alcohol consumption. In addition, the evidence for an inverse association between CVD risk and high sodium intake, high alcohol intake as well as consumption of trans-unsaturated fatty acids, myristic and palmitic acid was also considered to be convincing (WHO/FAO 2003). More recently, the Scientific Advisory Committee on Nutrition evaluated the evidence related to the consumption of carbohydrates and CVD risk (2015) and concluded that people should follow a dietary pattern that is based on carbohydrates from whole grains, pulses, potatoes as well as fruit and vegetables, but limit the amount from table sugar and rich sources of fruit sugar, such as preserves, spreads, fruit juice, confectionary, biscuits and sugar-sweetened beverages. With regards to fruit and vegetables, there is evidence to suggest that flavonoids might be cardioprotective (Macready 2014).

1.1.2.4 Emerging risk markers

In addition to the well-established CVD risk factors described above, several novel candidate risk factors have been suggested over the past few years, including presence of certain infectious agents (*Chlamydia pneumoniae*, *Helicobacter pylori*, *Porphyromonas gingivalis*, cytomegalovirus, HIV and influenza A virus) (Conelius 2014, Zimmer 2015), decreased levels of circulating progenitor cells (Hill 2003, Sen 2011, Donahue 2013, Werner 2005, Rigato 2016), increased levels of endothelial-(EMP) and platelet-derived microparticles (PMP), increased levels of inflammatory mediators and platelet hyperactivity. The latter three form part of this PhD project, hence evidence related to these is described in more detail in the following section.

1.1.2.4.1 Platelet hyperactivity

The European Food Safety Authority (EFSA) considers that "maintaining normal platelet aggregation" is a contributor to cardiovascular health: "Decreasing platelet aggregation in subjects with platelet activation during sustained exposure to the food/constituent (e.g. four weeks) would be a beneficial physiological effect" (EFSA Panel on Dietetic Products, Nutrition and Allergies 2011, EFSA Panel on Dietetic Products, Nutrition and Allergies 2009). While platelets play an essential role in hemostasis, they are also involved in atherosclerosis and thrombus formation, which may result in a stroke or myocardial infarction. The early stages of atherosclerosis are characterized by injured and/or inflamed endothelium, which facilitates platelet adhesion to endothelial cells (Figure 1). Following adhesion, platelets become activated and contribute to the development and progression of endothelial inflammation and atherosclerosis in several ways:

- Activated platelets secrete adhesive mediators, for example P-selectin and von Willebrand factor, that increase their adherence to circulating leucocytes, endothelial cells and other platelets. This results in increased recruitment and binding of circulating leucocytes to the endothelium and thus promotion of monocyte transmigration to the subendothelial space (Ruggeri 2002, Linden and Jackson 2010).
- Activated platelets secrete a number of proinflammatory compounds, such as platelet factor 4 (PF-4), regulated on activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein 1α (MIP-1α), epithelial neutrophil-activating protein 78 (ENA-78), CD40 ligand (CD40L) and P-selectin. These contribute to the inflammatory milieu by enhanced binding of LDL cholesterol to endothelial and smooth muscle cells, promotion of macrophage differentiation, and stimulation of monocytes as well as endothelial cells to produce inflammatory mediators (Vorchheimer and Becker 2006).
- Activated platelets secrete platelet-derived growth factor (PDGF) and transforming growth factor β that cause migration and proliferation of vascular smooth muscle cells (Vorchheimer and Becker 2006, Linden and Jackson 2010).

- Activated platelets shed microparticles (MP) from their surface, another potential promoter of the atherosclerotic process as described in more detail in section 1.1.2.4.2 (page 8) (Wang 2016).

As discussed in a recent review, acute cardiovascular events are triggered by a complex interaction between an unstable atherosclerotic plaque and increased procoagluant properties of platelets (Pasalic 2016) and there is some evidence about the predictive value of platelet activity on CVD risk. A prospective study following 149 myocardial infarction survivors for 5 years found that enhanced susceptibility of platelets to activate and aggregate (as measured by spontaneous platelet aggregation, e.g. aggregation observed on stirred platelet rich plasma (PRP) without added agonist) was associated with an increased risk of reoccurring acute coronary events and mortality (Trip 1990). Another prospective study followed 487 apparently healthy middle-aged men for 13.5 years. Platelet counts and adenosine diphosphate (ADP)-induced platelet aggregation in PRP were measured in a random subsample of 150 participants and both measures were related with CHD mortality (Thaulow 1991). This finding is in agreement with cross-sectional data from the Caerphilly Collaborative Heart Disease Study on 1,811 men, where an increased *ex vivo* ADP-induced platelet aggregation response in PRP was observed in patients with past myocardial infarction and patients with electrocardiographic evidence of ischemic heart disease compared to controls (no association was found between ADP-induced platelet aggregation and angina). There was also a significant relation between thrombin-induced aggregation with electrocardiographic evidence of ischemic heart disease, but not with past myocardial infarction (Elwood 1991). More recently, in 1,699 healthy individuals with a family history of early coronary artery disease, collagen-induced platelet aggregation measured by whole blood aggregometry was related with increased coronary artery disease in a 6 year follow-up period. In contrast, no effect was observed on ADP-induced aggregation (Qayyum 2015). However, there is also conflicting evidence. The Northwick Park Heart Study followed 1,369 men for 16 years. In a random subsample of 740 participants there

was no association between ADP- or adrenalin-induced platelet aggregation in PRP and the incidence of ischemic heart disease (Meade 1997). In summary, despite EFSA's view that "maintaining normal platelet aggregation" is a contributor to cardiovascular health (EFSA Panel on Dietetic Products, Nutrition and Allergies 2011, EFSA Panel on Dietetic Products, Nutrition and Allergies 2009) the data on the predictive value of platelet activity on CVD risk are inconclusive; this view is supported by several recent reviews in this area (Pasalic 2016, Sharma and Berger 2011). It is notable that the majority of studies in this area are quite dated and the application of different methods to assess platelet aggregation (varying agonists, concentrations) and different population groups and study durations make it difficult to draw a firm conclusion from this limited number of studies. More research is warranted to investigate the predictive value of platelet activity on CVD risk and improved standardization of the platelet function measurement protocol is considered essential (Pasalic 2016).

The effectiveness of antiplatelet medication on primary and secondary prevention of cardiovascular events has been demonstrated in several reviews and meta-analyses, particularly in secondary prevention and primary prevention of high risk individuals (Antithrombotic Trialists' Collaboration 2002, Antiplatelet Trialists' Collaboration 1994, Nemerovski 2012, Antithrombotic Trialists' Collaboration 2009, Kunutsor 2016). This highlights the crucial involvement of platelets in CVD pathogenesis. It is important to note, however, that antiplatelet therapy is not always recommended, especially in the case of primary CVD prevention in individuals at low CVD risk, as it also increases the risk of bleeding (Nemerovski 2012).

A variety of techniques are being applied in human intervention studies to assess platelet function (Bachmair 2014). The current gold standard is the measurement of *ex vivo* platelet aggregation using light transmission aggregometry in PRP and this methodology allows the measurement of the effects of several agonists on platelet aggregation, thus allowing the investigation of several activation pathways (Bachmair 2014). However, this technique has

received criticism for its relatively non-physiological nature, in particular as it is conducted in platelets separated from their physiological environment and platelets are stimulated under non-physiological low shear force (Ostertag 2010). There are also a number of other techniques that are frequently being used in the research community and these include:

- platelet aggregation in whole blood,
- measurement of platelet activation by quantification of platelet surface receptors using whole blood flow cytometry: P-selectin (a protein indicating platelet degranulation), activated fibrinogen receptor (GPIIb/IIIa) or platelet-monocyte conjugates,
- measurement of PMPs and
- measurement of *ex vivo* bleeding time using an automated platelet function analyzer (Bachmair 2014).

1.1.2.4.2 Number of circulating microparticles

The number of circulating MPs is another novel candidate risk marker for CVD. MPs are small plasma membrane vesicles that are shed from the surface of a variety of stimulated and/or apoptotic cells, for example leukocytes, erythrocytes, endothelial cells (EMP) and platelets (PMP) (VanWijk 2003). Only the latter two will be discussed in this section, although recent evidence suggests the involvement of further extracellular vesicles in atherogenesis and thrombogenesis as reviewed elsewhere (Chistiakov 2015, Suades 2015). PMPs and EMPs have been suggested to serve as biomarkers of vascular injury and inflammation (Martinez 2011). With regards to PMP, as they are shed upon platelet activation, it has been suggested that they might also serve as a general marker for platelet activation (Bachmair 2014).

It is believed that EMPs and PMPs contribute to the pathogenesis of CVD in a variety of ways (Martinez 2011):

- Firstly, PMPs and EMPs have been shown to act in a pro-inflammatory manner by increasing the adhesion of circulating monocytes to the endothelium through upregulating adhesion molecules and by stimulating the release of cytokines by endothelial cells (VanWijk 2003).
- Secondly, PMPs and EMPs have also been suggested to promote coagulation by possessing a procoagluant negatively charged phospholipid surface, via initiating and promoting thrombin generation or indirectly through the presence of P-selectin on the PMP surface, which could stimulate monocytic tissue factor expression (VanWijk 2003).
- Lastly, EMPs might be involved in inducing and maintaining endothelial dysfunction by limiting nitric oxide (NO) production and thus diminishing endothelium-dependent relaxation (VanWijk 2003, Heiss 2015).

Supporting evidence for a role of PMPs and EMPs in CVD pathogenesis comes from studies demonstrating that increased levels of circulating PMPs and EMPs are found in patients with CVD risk factors, such as obesity (La Vignera 2012, McFarlin 2015) and hypertension (Mallat 2000) and they are increased in patients with acute coronary syndromes (Mallat 2000) and after acute myocardial infarction (Boulanger 2006).

Despite these detrimental properties that have been linked to EMPs and PMPs, it has been suggested that some MPs can have beneficial properties through acting on promotion of endothelial progenitor cell differentiation, increasing NO bioavailability and inducing angiogenesis. However, research into this area is only just emerging (Martinez 2011).

1.1.2.4.3 Inflammation and immunity

Inflammation is present in all stages of atherosclerosis and there are several cells and mediators involved (Ross 1999, Libby 2012, Libby 2013, Libby and Hansson 2015, Zimmer 2015,

Yaqoob and Ferns 2005, Ait-Oufella 2011). As such, several markers of inflammation have been suggested as predictors of CVD risk, including C-reactive protein (CRP), fibrinogen, serum amyloid A, albumin, leucocyte count, erythrocyte sedimentation rate, circulating immune complexes, growth factors, soluble adhesion molecules, heat-shock proteins, phospholipase A2, paraoxonase-1 and circulating levels of cytokines (Yaqoob and Ferns 2005). Whilst cytokines are important messenger molecules in the immune system, a number of inflammatory cytokines, in particular TNF-α, IL-1β, IL-6 and IL-8 also contribute to the development of atherosclerosis and plaque destabilization in several ways:

- Stimulation of lymphocyte and monocyte migration into the subendothelial space through enhanced expression of endothelial adhesion molecules and chemokines.
- Stimulation of leucocyte activation and the formation of foam cells in the subendothelium through upregulation of scavenger receptors and stimulation of cell-mediated oxidation.
- Contribution to plaque destabilization through (i) stimulation of smooth muscle cell and macrophage apoptosis, resulting in enlargement of the necrotic core and thinning of the fibrous cap, and through (ii) matrix degradation via decreased collagen production and modulated expression of matrix metalloproteinases.
- Increase in the endothelium's prothrombotic potential through increased tissue procoagulant activity and decreased activity of anticoagulant mediators (thrombomodulin and protein C receptor) (Ait-Oufella 2011).

Consequently, inappropriate or excessive production of TNF-α, IL-1β, IL-6 and IL-8 has been linked with the pathogenesis of atherosclerosis and a number of other chronic inflammatory diseases (Yaqoob and Calder 2011). IL-10, on the other hand, is a predominantly anti-inflammatory cytokine and would be expected to be associated with reduced atherosclerosis by suppressing macrophage activation and inhibiting several pro-inflammatory cytokines, chemokines and growth factors (Ait-Oufella 2011).

Convincing evidence on the involvement of cytokines in atherosclerosis comes from numerous mouse experiments, where the blockage of proinflammatory cytokines is associated with reduced development and progression of atherosclerotic plaque (Ait-Oufella 2011). In line with this, a recent meta-analysis of prospective studies in humans demonstrated an association between circulating concentrations of the inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α and CHD risk. This association was independent of other risk factors (Kaptoge 2014).

A link between inflammation and CVD risk is also indicated in pharmacological trials. Statin therapy not only lowers LDL cholesterol and decreases CVD risk, but also reduces inflammation (as determined by CRP levels). It is believed that both, the anti-inflammatory and lipid-lowering effect contribute to the CVD benefits (Ridker 2009, Libby 2012). Other pharmacological trials demonstrate that anti-TNF-α therapy results in reduction of cardiovascular events (Ait-Oufella 2011). However, complete blockage of TNF-α might not be an ideal solution for long-term treatment due to its essential role in the immune system. There are currently other anti-inflammatory medications under investigation in this area, such as low-dose methotrexate or IL-1β inhibitors. As these have anti-inflammatory properties, but no effect on blood lipid, these trials will verify the effect of anti-inflammatory treatment on cardiovascular outcomes (Libby 2012, Libby 2013).

1.2 Polyphenols

Polyphenols are secondary metabolites that are synthesized by a wide variety of plants. They fulfil a number of functions in plants, including protection against excessive light, attraction of pollinating insects and seed-dispersing animals, protection against harmful insects and microbes, stimulation of nitrogen-fixing nodules and as allelopathic agents. Polyphenols can

be classified according to their chemical structure into non-flavonoids and flavonoids and only the latter group will be described in more detail as the present thesis is concerned with the health benefits of a flavonoid subclass (Crozier 2009, Crozier 2006).

Flavonoids share a common chemical structure of two aromatic rings that are connected by a three carbon bridge (Figure 2). This 15-carbon structure can be abbreviated C6-C3-C6. The flavonoid subclasses with most significant dietary relevance are flavonols, flavones, isoflavones, flavanones, flavan-3-ols and anthocyanidins and they differ in their C-ring substituents (Figure 2) (Crozier 2009). Flavonoids are generally found as monomers or polymers in fruits, vegetables and plant based food products such as tea, wine and cocoa (Crozier 2009).

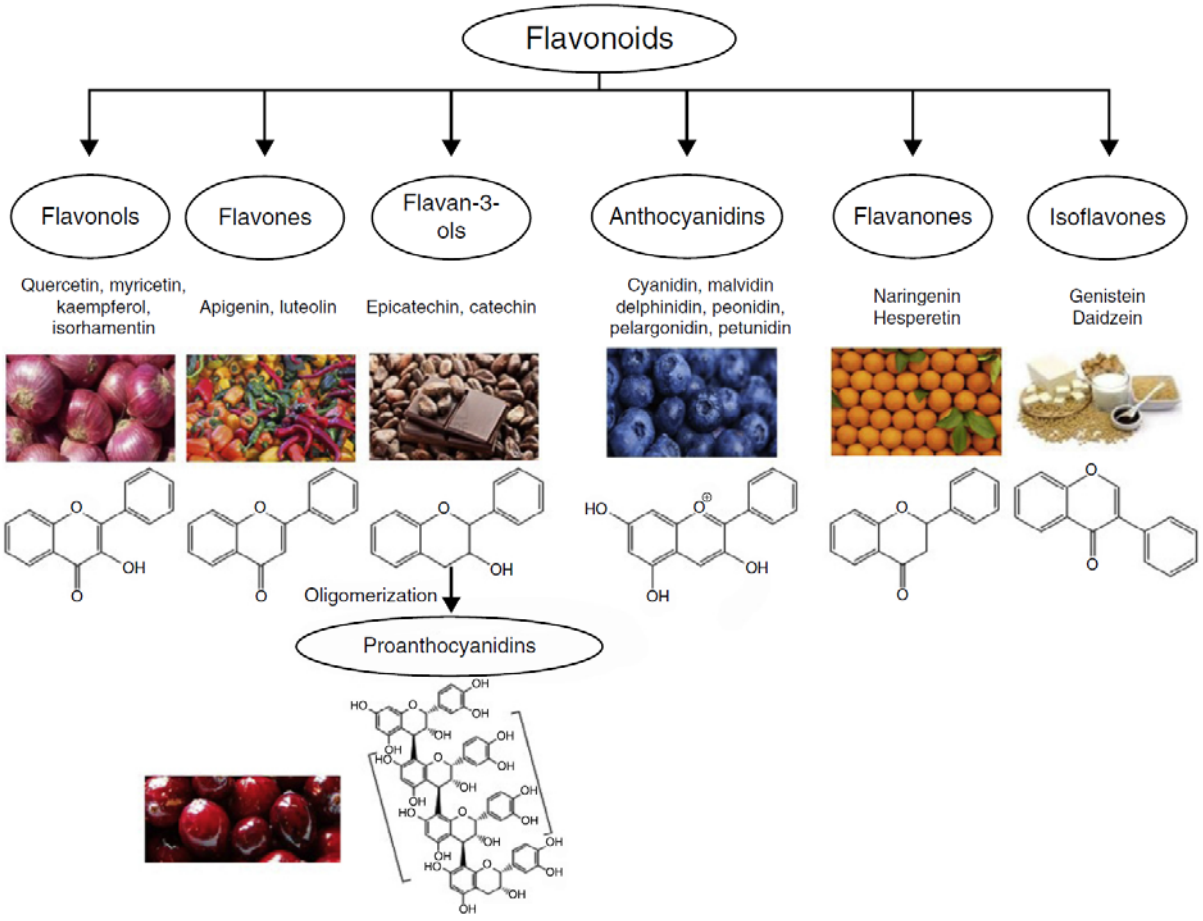
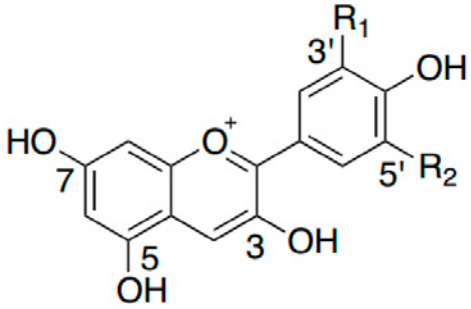


Figure 2 Dietary flavonoids: Main classes, subclasses, chemical structures and examples of dietary sources (Feliciano 2015).

1.2.1 Anthocyanidins and anthocyanins (ACNs)

Anthocyanidins are a class of flavonoids that are responsible for the blue, red and purple color of many fruits and vegetables. Among particularly rich dietary sources are berry fruits, but also some vegetables, such as red cabbage, red onion, red radish and aubergines (Table 1) (Neveu 2010, Koponen 2007). The most common dietary anthocyanidins are cyanidin (Cy), delphinidin (Dp), malvidin (Mv), pelargonidin (Pg), peonidin (Pn) and petunidin (Pt) and these differ in their substitutions on the B-ring (Figure 3) (Crozier 2006). Anthocyanidins are generally present in plants conjugated to sugars (Table 1), these glycosylated anthocyanidins are called anthocyanins (ACNs) (Crozier 2006). Most ACN-rich foods contain a number of different ACNs (Table 1), sometimes based on one anthocyanidin, such as black elderberry, blackberry, chokeberry or raspberry, whilst other dietary sources contain ACNs, which are based on two or more types of anthocyanidins, for example blackcurrant and blueberry. In recent years, ACNs have attracted considerable interest as dietary constituents that may provide a variety of health benefits to humans, including CVD prevention, obesity control, alleviation of diabetes and improvement of vision and memory (reviewed in Tsuda 2012, Rodriguez-Mateos 2014a) and the current thesis particularly focusses on their potential to exert effects on the immune and cardiovascular system.



Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malviidin	OCH_3	OCH_3

Figure 3 Structures of main dietary anthocyanidins (Crozier 2006).

Table 1 Anthocyanins (amount and type) in different fruits, vegetables and beverages

Food/beverage source		ACN content *(mg/100 g or mg/100 mL)*	Main ACNs *(type)*	*(mg/100 g)*
Fruit	Black elderberry	1,316[a]	Cy-3,5-diglc	17
			Cy-3-glc	794
			Cy-3-rut	6
			Cy-3-sam	463
			Cy-3-sam-5-glc	34
	Blackberry	147	Cy-3-(6”-dioxalyl-glc)	11
			Cy-3-glc	139
			Cy-3-rut	9
			Cy-3-xyl	10
	Blackcurrant	225	Cy-3-glc	25
			Cy-3-rut	161
			Dp-3-glc	87
			Dp-3-rut	305
			Pt-3-(6”-p-coumaroyl-glc)	5
	Blueberry (highbush)	164	Cy-3-gal	5
			Dp-3-ara	14
			Dp-3-gal	20
			Dp-3-glc	8
			Mv-3-ara	13
			Mv-3-gal	17
			Mv-3-glc	11
			Pt-3-ara	9
			Pt-3-gal	13
			Pt-3-glc	6
	Blueberry (lowbush)	149	Cy-3-gal	8
			Cy-3-glc	8
			Dp-3-(6”-acetyl-glc)	6
			Dp-3-ara	7
			Dp-3-gal	16
			Dp-3-glc	15
			Mv-3-(6”-acetyl-gal)	6
			Mv-3-(6”-acetyl-glc)	15
			Mv-3-ara	8
			Mv-3-gal	21
			Mv-3-glc	26
			Pn-3-glc	6
			Pt-3-gal	9
			Pt-3-glc	11
	Cherry (sour)	49	Cy-3-glc-rut	44
			Cy-3-rut	7
	Cherry (sweet)	32	Cy-3-glc	19
			Cy-3-rut	143
			Pn-3-rut	7

Food/beverage source		ACN content *(mg/100 g or mg/100 mL)*	Main ACNs *(type)*	*(mg/100 g)*
Fruit	Chokeberry	444	Cy-3-ara Cy-3-gal Cy-3-glc Cy-3-xyl	253 558 20 46
	Raspberry	43	Cy-3-glc Cy-3-glc-rut Cy-3-rut Cy-3-sop	15 7 5 38
	Redcurrant	26	Cy-3-sam Cy-3-xylosyl-rut	9 11
	Strawberry	73 [a]	Pg-3-(6”-succinyl-glc) Pg-3-glc	10 47
Vegetable	Aubergine (Koponen 2007)	8 [a]	Dp glycosides (subgroup analysis not conducted)	8
	Red cabbage (Koponen 2007)	75 [a]	Cy glycosides (subgroup analysis not conducted)	75
	Red onion	9 [a]	Dp-3-glc-glc	7
	Red radish (Koponen 2007)	32 [a]	Pg glycosides (subgroup analysis not conducted)	32
Alcoholic Beverage	Red wine	22	Mv-3-glc	10

This is not an exhaustive list. Total ACN content obtained by pH differential method unless otherwise indicated. [a] Total ACN content calculated as sum of the content of individual ACNs obtained by chromatography (where data from pH differential method not available). Data on ACN subclasses obtained by chromatography and only listed where the content exceeded 5 mg/100 g. The color of different anthocyanidins at acidic pH is indicated in the latter two columns. Complied using Phenolexplorer database (Neveu 2010, accessed 23/06/16), unless otherwise indicated. ACN, anthocyanin; ara, arabinoside; Cy, cyanidin; diglc, diglucoside; Dp, delphinidin; gal, galactoside; glc, glucoside; Mv, malvidin; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; rut, rutinoside; sam, sambubioside; sop, sophoroside; xyl, xyloside.

1.2.2 ACN bioavailability

Studies on ACN bioavailability provide critical information regarding the concentration and nature of compounds occurring in circulation upon ACN consumption. This data is vital to inform the design of cell culture experiments investigating the bioactivity of ACNs.

Early studies concerned with the ACN bioavailability in humans reported that ACNs appear in the blood within a few minutes upon consumption, but are characterized by a low bioavailability. Many studies reported recoveries in blood of less than 1% of the ingested dose, with maximum blood concentrations in the range of 1-270 nmol/L.

Several early bioavailability studies have only detected the unmetabolised parent ACN glycosides in plasma and urine, such as following consumption of hibiscus extract (Frank 2005a), red fruit extract (Miyazawa 1999), elderberry (Frank 2005b, Netzel 2005, Cao 2001, Murkovic 2000, Cao and Prior 1999), blackcurrant ACN concentrate (Matsumoto 2001, McGhie 2003), whole blueberries (Wu 2002), blueberry powder (Mazza 2002), blueberry extract (McGhie 2003), cranberries (Milbury 2010, Ohnishi 2006), purple sweet potato beverage (Oki 2006, Harada 2004), purple carrot juice (Charron 2009) and purple carrot sticks (Kurilich 2005).

Furthermore, additional early evidence indicated that ACNs degrade to their respective phenolic acid and aldehyde constituents upon consumption and that these may play a significant role in the fate of ACNs upon ingestion. As such, protocatechuic acid (PCA), 4-hydroxybenzoic acid, gallic acid, syringic acid and vanillic acid were identified as major degradants of cyanidin-3-O-glucoside (Cy-3-glc), pelargonidin-3-O-glucoside (Pg-3-glc), delphinidin-3-O-glucoside, malvidin-3-O-glucoside, peonidin-3-O-glucoside, respectively (Vitaglione 2007, Azzini 2010, Fleschhut 2006). For example, upon consumption of blood orange juice rich in Cy-3-glc and Cy-3-(6-malonyl)-glc, PCA was identified as the major ACN metabolite in serum and feces: the maximal concentration in blood for Cy-3-glc was 1.9 nm, whereas the value for PCA was approximately 250-fold higher (492 nM). It was calculated that serum PCA accounted for about 70% of the ACN intake (Vitaglione 2007).

A limited number of early human studies also indicated that the ACN parent compounds as well as its phenolic acid and aldehyde degradation products can undergo conjugation to methyl, glucuronide, and sulfate metabolites (Mullen 2008, Kay 2004, Kay 2005, Wu 2002, Wiczkowski 2010) but these reports provide conflicting information regarding the nature and prevalence of the resulting metabolites.

Novel insights on ACN bioavailability, in particular related to Cy glycosides, have been obtained in the last few years facilitated by optimized analytical methods for a thorough analysis of ACN metabolites in clinical samples and by the use of more sensitive instrumentation. Detailed accounts of these optimized methodologies are available in the public domain (de Ferrars 2014b, Feliciano 2016b). A key study made use of an isotope-labelled ACN (Cy-3-glc) and revealed that ACNs are much more bioavailable and more extensively metabolized than previously believed and that metabolites are still present at ≤48 h post consumption (Czank 2013, de Ferrars 2014c). The relative bioavailability of the $^{13}C_5$-labelled Cy-3-glc was established as 12%, with 5% of the ^{13}C dose being recovered in urine, 7% in breath and 32% in feces. A total of 17 ^{13}C compounds were identified in serum (Table 2), including a number of metabolites alongside the unmetabolised parent ACN, although the latter was identified as a minor constituent with a low C_{max} of 0.14 µmol/L and a short half-life of 0.4 h. As such, the predominant compounds recovered in serum were metabolites and these were identified as phase II conjugates of PCA (several methylated, glucuronidated and sulphated compounds, combined C_{max} of 2.35 µmol/L, in this subgroup vanillic acid appeared as the main metabolite, although its presence in serum was only detected in 2 out of 8 volunteers), hippuric acid (C_{max} of 1.96 µmol/L), ferulic acid (C_{max} of 0.94 µmol/L) and Cy-3-glc degradation products (combined C_{max} of 0.73 µmol/L) (Table 2). While the parent ACN reached its maximal plasma concentration at 1.81 h post-consumption, the metabolites peaked between 6-16 h post-consumption (Czank 2013, de Ferrars 2014c).

The same research group has reported extensive metabolism of elderberry ACNs, with the parent ACNs as well as 19 metabolites being reported in plasma samples (de Ferrars 2014a).

Table 2 Serum pharmacokinetics of Cy-3-glc and its metabolites established in an isotope-labelled human intervention study

Compound group	Compound	C_{max} ($\mu mol/L$)	T_{max} (h)	Commercially available?
Parent ACN	Cy-3-glc	0.14	1.8	Yes
Degradants	PCA	0.15	3.3	Yes
	PGA	0.58	2.8	Yes
Phase II conjugates of PCA	PCA-3-glucuronide	0.01	2.7	No
	PCA-4-glucuronide	0.07	3.8	No
	PCA-3-sulfate	0.16	11.4	No
	PCA-4-sulfate			
	vanillic acid	1.86	12.5	Yes
	isovanillic acid	0.20	2.0	Yes
	vanillic acid-glucuronide	0.02	4.8	No
	isovanillic acid-glucuronide	0.04	4.3	No
	vanillic acid-sulfate	0.43	30.1	No
	isovanillic acid-sulfate	n.q.	n.q.	No
	methyl 3,4-dihydroxybenzoate	0.01	8.4	Yes
	ferulic acid	0.94	11.29	Yes
	hippuric acid	1.96	15.69	Yes

(de Ferrars 2014c, Czank 2013) C_{max}, maximum concentration; Cy-3-glc, cyanidin-3-O-glucoside; n.q., not quantified; PCA, protocatechuic acid; PGA, phloroglucinaldehyde; T_{max}, time of maximum concentration.

Blueberry polyphenols have also been reported to undergo extensive metabolism upon consumption. However, blueberries do not only contain ACNs, but also proanthocyanidins, cholorogenic acids and flavonols and it is therefore difficult to relate plasma phenolics to their originating parent compounds (Rodriguez-Mateos 2013, Rodriguez-Mateos 2014b, Feliciano 2016a, Rodriguez-Mateos 2016).

1.3 ACNs and CVD risk

There is growing evidence that ACNs can modify CVD risk (markers) and this area has been investigated in epidemiological studies (summarized in section 1.3.1) as well as acute and chronic human intervention studies (summarized in section 1.3.2 and 1.3.3 respectively).

1.3.1 Epidemiological studies on ACNs and CVD risk

Several epidemiological studies have investigated the relationship between ACN intake and the risk of developing CVD. In the Nurses' Health Study II, a prospective cohort study following 93,600 young and middle-aged women for 18 years, a higher intake of ACNs was associated with a reduced risk of myocardial infarction (Cassidy 2013), despite a narrow range of ACN intakes between quintiles and a low ACN intake even in the highest quintile (2 to 35 mg/d). A risk reduction of 32% was observed comparing the highest versus the lowest quintile of ACN intake (hazard ratio: 0.68; 95% CI: 0.49, 0.96; *P*-trend = 0.047) and a 17% decrease in the risk of myocardial infarction was detected for every 15 mg increase in ACN consumption. It is noteworthy to point out that the reduction in risk was independent of established dietary/lifestyle CVD risk factors, including smoking, body mass index (BMI), and fruit and vegetable intake. The investigators also examined the association on food level and observed that combined intake of strawberries and blueberries tended to be associated with a decreased risk when comparing those who consume >3 weekly servings versus those with lower intake (Cassidy 2013). This is in line with data from the Cancer Prevention Study II, a prospective cohort study following 98,469 men and women for 7 years, where a higher intake of anthocyanidins was associated with a reduced risk of CVD mortality (relative risk: 0.86; 95% CI: 0.83, 0.99; *P*-trend = 0.032). However, when the data were stratified by gender, this effect was not significant for men (relative risk: 0.91; 95% CI: 0.77, 1.06; *P*-trend = 0.3)

(McCullough 2012). Further epidemiological evidence for a cardioprotective effect comes from the Iowa Women's Health Study, a prospective cohort study following 34,489 postmenopausal women for 16 years, where a higher intake of anthocyanidins was associated with a reduced risk of total CVD and CHD mortality (multivariate-adjusted rate ratio: 0.91; 95% CI: 0.83, 0.99; *P*-trend = 0.032 and multivariate-adjusted rate ratio: 0.88; 95% CI: 0.78, 0.99; *P*-trend = 0.031, respectively). On a food level, an inverse association between strawberry intake and total CVD-related mortality was observed in the multivariate-adjusted model (rate ratio: 0.91; 95% CI: 0.82, 1.00; *P*-trend = 0.046), but no significant association was observed with CHD-related mortality. Associations were also evaluated for blueberry consumption and whilst they were significant for total CVD-related mortality in the age- and energy-adjusted model (*P*-trend = 0.008), the significance disappeared after multivariate adjustment. The same was true for associations with CHD mortality (Mink 2007). Taken together, even though blueberries and strawberries were identified as major anthocyanidin sources in this population, these studies indicate that it is the totality of anthocyanidin-rich foods that is associated with reduced CHD mortality risk. Despite the three encouraging epidemiological studies, there was no significant association between ACN intake and incidence of total CVD or CHD in the Framingham Offspring Cohort in middle-aged and older men and women followed for 14.9 years (Jacques 2015). It is possible that the relatively small participant number contributed to the nonsignificant finding. This study followed 2,880 people, which is more than 10-fold less compared to the other epidemiological studies described above.

Fewer epidemiological studies have investigated the relationship between ACN intake and the risk of developing stroke, with less encouraging results compared to the CHD and total CVD data. No association was found between anthocyanidin intake and stroke mortality in the Iowa Women's Health Study, a prospective cohort study following 34,489 postmenopausal women for 16 years (multivariate-adjusted rate ratio: 1.01; 95% CI: 0.83, 1.24; *P*-trend = 0.896).

Similarly, no significant association between ACN intake and stroke incidence was observed in the Nurses' Health Study on 69,622 women with a 14 year follow-up period. It should be pointed out, however, that the range of ACN intakes between the different quintiles was very narrow with only a 10.7 mg/d difference between the extreme quintiles (5.8 versus 16.5 mg/d) (Cassidy 2012) and this might have contributed to the finding. To provide an example on the food level, 10 mg of ACNs are contained in approximately five blueberries (7 g) or one large strawberry (14 g), the main ACN sources in this study, and one can argue that it is questionable whether such a small amount can be accurately assessed using food frequency questionnaires.

In addition to epidemiological studies examining associations with clinical endpoints, there are also studies which consistently reported an inverse association between ACN intake and risk factors of CVD, in particular arterial stiffness (Jennings 2012), high blood pressure (BP) (Jennings 2012, Cassidy 2011) and CRP as a marker of inflammation (Chun 2008).

In summary, most, but not all, epidemiological studies suggest a protective effect of ACNs against total CVD and CHD, while the evidence for stroke is less convincing. As such, one could generate the hypothesis of a protective effect of ACNs on CVD endpoints, in particular CHD, and CVD risk markers. However, there are a number of limitations inherent to epidemiological studies, for example the accurate estimation of food intake per se, the accurate estimation of ACN intakes from dietary information, natural variations in ACN contents in foods and the difficulty to control for other confounding factors. For example, in Mink's study, subjects with a higher flavonoid intake had on average a lower BMI, lower waist-hip-ratio, exercised more, were less likely to smoke, were more likely to be users of multivitamins and had a better educational status (Mink 2007). Whilst studies usually adjust for confounding factors, this is not always the case. In the study by Mink et al, the significant inverse association between blueberry intake and total CVD-related mortality disappeared after adjustment for age, energy intake, marital status, education, BP, diabetes, BMI, waist-to-hip ratio, physical activity,

smoking and estrogen use, suggesting that the observed association may be a consequence of those factors. Thus, whilst epidemiological studies are of great value in terms of hypothesis generation, well-controlled randomized controlled trials (RCTs) are required to examine the causal link between ACN consumption and CVD and their risk markers. Few studies have been conducted in this area and these are described in the next section.

1.3.2 Acute human intervention trials on ACNs and selected markers of CVD risk

The cardioprotective properties of ACNs observed in epidemiological studies could be mediated by a variety of mechanisms including:

- direct scavenging effect against free radicals or reactive oxygen species in general,
- inhibition of reactive oxygen species-generating enzymes,
- modulation of vasodilatory action,
- modulation of platelet activation and aggregation and
- modulation of cytokine, adhesion molecule and chemokine production.

This section is confined to human intervention studies examining the effects of ACNs on vascular function, arterial stiffness, BP, platelet function, MPs and inflammatory markers; acute studies are reviewed in section 1.3.2 and chronic studies in section 1.3.3.

1.3.2.1 Effects of ACNs on vascular function, arterial stiffness and blood pressure

A summary of the studies is presented in Table 3. The acute effect of ACN-rich interventions on vascular function was investigated in eight studies with mixed findings; the majority of studies were conducted using blueberry-based interventions, but the effect of a boysenberry beverage, an açai smoothie, a blackcurrant juice drink and purified ACNs was also investigated.

The majority of studies employed the gold standard technique for the assessment of vascular function (flow-mediated dilation, FMD), but EndoPAT and laser Doppler imaging (LDI) were also used. A crossover RCT in 10 healthy men reported a bimodal increase in FMD following the ingestion of various doses of a blueberry drink: The first FMD peak occurred 1-2 h post-consumption and was accompanied with the appearance of metabolites assumed to originate from the blueberry-derived ACNs and chlorogenic acid (ferulic acid, vanillic acid, isoferulic acid, benzoic acid, 2-hydroxybenzoic acid, and caffeic acid), while the second peak occurred 6 h post-consumption and was accompanied with metabolites assumed to be produced by the gut microbiota (hippuric acid, hydroxyhippuric acid, 3-(3-hydroxy)-phenylpropionic acid, dehydroferulic acid, homovanillic acid, and 2,5-dihydroxybenzoic acid) (Rodriguez-Mateos 2013, Rodriguez-Mateos 2014a). The FMD improvement was also linked with inhibited neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, which in turn would be expected to increase endothelial NO concentrations (Rodriguez-Mateos 2013). In a subsequent study, the improvements in FMD response were demonstrated to be dose-dependent between 319 and 766 mg total polyphenol (TP) doses (129 to 310 mg ACNs respectively), followed by a plateau in the response with higher intakes (up to 1,791 mg TP) (Rodriguez-Mateos 2013). Subsequently, a carefully processed blueberry-containing baked product was demonstrated to result in a similar biphasic FMD response in comparison to a blueberry drink prepared using the same amount of freeze-dried blueberry powder, but the maximum increase in FMD was delayed with the baked product from 1 to 2 h and the authors propose that this was probably related to slower gastric passage of the solid baked product (Rodriguez-Mateos 2014b). Acute improvements in FMD were also demonstrated following consumption of 320 mg purified ACNs derived from bilberry and blackcurrant in a study with 12 hypercholesteraemic subjects (Zhu 2011) and following consumption of an açai smoothie containing 943 mg ACNs in overweight men (Alqurashi 2016). In contrast, there was no

significant effect on FMD in a small, open-label, uncontrolled study with a boysenberry drink containing 351 mg TP (Matsusima 2013). Similarly, no acute effect was observed following blueberry consumption in 10 healthy young males using the EndoPAT technique (Del Bo 2013) and following consumption of a blackcurrant juice drink in 20 healthy males and females using the LDI technique (Jin 2011).

Discrepant findings could have arisen due to different techniques applied to assess vascular function, as well as differences in the nature and dose of interventions. It is striking, for example, that the blackcurrant drink in Jin's study (2011), which was negative, provided a much lower dose of ACNs compared to studies that did report a significant improvement in vascular function (Table 3). Regarding different techniques to assess vascular function, FMD is generally considered the most sensitive technique to assess vascular function (Onkelinx 2012) and CVD risk (Lind 2013). As such, this might have contributed to discrepant findings. In support of this hypothesis, the majority of studies applying the FMD technique demonstrated significant effects of ACN-rich interventions on vascular function, whilst the two studies applying alternative techniques found no effect (Table 3). However, this hypothesis is based on a very small number of studies that not only differ in the techniques to assess vascular function, but also in several other methodological aspects (Table 3). Furthermore, some studies were of overall questionable quality, such as Matsusima's study (2013), which had no control group and only 6 volunteers, inviting speculation that it might be underpowered.

Regarding BP, most studies showed no acute effect following consumption of blueberry beverages (Rodriguez-Mateos 2013, Rodriguez-Mateos 2014b), a blueberry bun (Rodriguez-Mateos 2014b), an açai smoothie (Alqurashi 2016) or a boysenberry beverage (Matsusima 2013) as summarized in Table 3. Only one recent study showed an improvement in both systolic BP (SBP) and diastolic BP (DBP) following a cherry juice intervention (Kent 2016). As such, the

effect of ACN-rich interventions on BP requires confirmation in subsequent studies before a conclusion can be reached.

The acute effect of ACN-rich interventions on arterial stiffness was only investigated in one study and there were no significant effects of acute consumption of a blueberry beverage on pulse wave analysis (PWA), pulse wave velocity (PWV), digital volume pulse stiffness index (DVP-SI) or digital volume pulse reflection index (DVP-RI) (Rodriguez-Mateos 2013), but this also requires confirmation in subsequent studies.

Table 3 Human intervention studies investigating the acute effects of ACN-rich interventions on vascular function, arterial stiffness, blood pressure, platelet function and circulating EMPs and PMPs

Outcome	Intervention	Polyphenol dose (mg) / serving	Subjects	Study design	Timepoints	Results	Reference
Vascular function, arterial stiffness and blood pressure	Blueberries	not measured	n = 20, chronic smokers, mean age: 28 y	RCT, parallel	B, 1 h	⇔ BP ⇔ angiotensin converting enzyme activity	McAnulty (2005)
	Blackcurrant juice drink	31 Dp glycosides, 20 Cy glycosides	n = 20, M+F, healthy, mean age: 45 y	RCT, DB, crossover	B, 2 h	⇔ LDI	Jin (2011)
	Purified bilberry and blackcurrant ACN capsules	320 ACN: 59% Dp glycosides, 33% Cy glycosides	n = 12, M+F, hypercholesteremic, age: 40-65 y	RCT, crossover	B, 1, 2, 4 h	↑ FMD at 1 & 2 h ⇔ FMD at 4 h ↑ cGMP at 1 & 2 h ⇔ cGMP at 4 h	Zhu (2011)
	Blueberries	727 TP, 348 ACN, 90 CA	n = 10, M, healthy, mean age: 21 y	RCT, crossover	B, 1 h	⇔ EndoPAT	Del Bo (2013)
	Boysenberry beverage	351 TP, 4.65 flavanols	n = 6, M+F, SBP: 120-172 mm Hg, age: 34-68 y	open-label, uncontrolled	B, 1, 2, 3.5 h	⇔ FMD ⇔ BP	Matsusima (2013)
	Blueberry beverage	766-1,791 TP, 310-724 ACN, 137-320 Pro, 273-637 CA	n = 10, M, healthy, age: 18-40 y	RCT, DB, crossover	B, 1, 2, 4, 6 h	↑ FMD at 1, 2 & 6 h ⇔ FMD at 4 h ⇔ BP ⇔ PWA and PWV ⇔ DVP-SI and DVP-RI	Rodriguez-Mateos (2013)
	Blueberry beverage	319-1,791 TP, 129-724 ACN, 57-320 Pro, 114-637 CA	n = 11, M, healthy, age: 18-40 y	RCT, DB, crossover	B, 1 h	↑ FMD (dose-dependent effect between 319-766 mg TP and plateaued response at higher concentrations)	Rodriguez-Mateos (2013)
	Blueberry, 2 interventions: - beverage - bun	beverage / bun: 692 / 637 TP, 339 / 196 ACN, 111 / 140 Pro, 179 / 221 CA	n = 10, M, healthy, age: 18-40 y	RCT, crossover	B, 1, 2, 4, 6 h	↑ FMD at 1, 2 & 6 h with both interventions (maximum FMD increase at 1 h [drink], 2 h [bun]) ⇔ FMD at 4 h ⇔ BP	Rodriguez-Mateos (2014b)
	Blueberries	69 ACN/100 mL	n = 13 (6 young [18-35 y] & 7 old [55 y+])	uncontrolled	B, 2, 6 h	**Single 300 mL serving:** ↓ SBP, DBP + HR at 2 h ⇔ SBP, DBP + HR at 6 h **3x100 mL serving:** ⇔ SBP, DBP + HR at 2 & 6 h	Kent (2016)
	Açai smoothie	694 TP 493 ACN 173 gallic acid	n = 23, M, healthy, overweight mean age: 46 y	RCT, DB, crossover	B, 2, 4, 6 h	↑ FMD at 2, 4 & 6 h ⇔ BP	Alqurashi (2016)

Outcome	Intervention	Polyphenol dose (mg) / serving	Subjects	Study design	Timepoints	Results	Reference
Platelet function	Purple grape juice	1017 TP (mainly flavonols, ACNs and Pro, but quantification on subclasses not provided)	n = 10, M+F, healthy, mean age: 42 y	acute-on-chronic, uncontrolled	2 h (juice consumed daily for 7-10 d before study day)	↓ collagen-induced platelet aggregation - by 77% compared to baseline (from 17.9 to 4.0) with 1 mg/L - by 21% compared to baseline (from 24.5 to 19.3) with 12.5 mg/L ↔ ADP- or thrombin-induced platelet aggregation	Keevil (2000)[1]
	Wine grape extract capsules	800 TP, 137 ACN, 9.6 PA, 4.2 catechin, 0.9 flavonols, 0.2 stillbenes	n = 35, M, healthy, mean age: 35 y	acute-on-chronic, RCT, DB, crossover	1.75 h after breakfast, 3.75 h after lunch (capsules consumed daily for 2 wks before study day)	↔ closure time, but a trend for improvement after lunch (P=0.06) ↔ platelet aggregation, but a trend for inhibition of ADP-induced aggregation after lunch (P<0.1)	van Mierlo (2010)[2]
Circulating EMPs and PMPs	Cocoa beverage	897 TP	n = 30, M+F, healthy, age: 24-50 y	RCT, parallel	B, 2, 6 h	↓ EMPs at 2 & 6 h	Rein (2000)[3]

ACN, anthocyanins; B, baseline; BP, blood pressure; CA, chlorogenic acid; cGMP, cyclic guanosine monophosphate; Cy, cyanidin; DB, double-blind; DBP, diastolic blood pressure; Dp, delphinidin; DVP-RI, digital volume pulse reflection index; DVP-SI, digital volume pulse stiffness index; EMP, endothelial-derived microparticles; FMD, flow-mediated dilation; HR, heart rate; LDI, laser Doppler imaging; PA, phenolic acids; PMP, platelet-derived microparticles; Pro, procyanidins; PWA, pulse wave analysis; PWV, pulse wave velocity; RCT, randomized controlled trial; SBP, systolic blood pressure; TP, total polyphenols.

[1] method for platelet function measurement: impedance aggregometry in whole blood (activators: collagen (1 mg/L, 12.5 mg/L), ADP & thrombin (concentration not provided for latter two))
[2] method for platelet function measurement: platelet function analyzer 100 (activators: epinephrine/collagen and ADP/collagen) and light transmission aggregometry in PRP (activators: ADP (10 μM) and collagen (concentration not provided)
[3] method for PMP measurement: Flow cytometry: PMP: CD42a+

1.3.2.2 Effects of ACNs on platelet function

To the best of our knowledge, only two studies have investigated the acute effects of ACN-rich interventions on platelet function (Table 3) (van Mierlo 2010, Keevil 2000). However, both studies were focused on acute-on-chronic effects and thus RCTs investigating "true" acute effects are lacking. In van Mierlo's study (2010), no significant effect on platelet function was observed 1.75 h post-consumption of an encapsulated wine grape extract, which was consumed together with breakfast. Later in the study day, a trend for improved epinephrine/collagen-stimulated closure time (P=0.06) and a trend for inhibited ADP-induced platelet aggregation (P<0.1) was demonstrated (Table 3) at 3.75 h following consumption of a second portion of the encapsulated wine grape extract together with a high-fat lunch. However, the placebo capsules only contained cellulose, so the study design does not allow identification of the compounds responsible for the trend (van Mierlo 2010). In contrast, a dramatic inhibition in collagen-induced platelet aggregation was demonstrated upon consumption of purple grape juice: platelet aggregation was reduced by 77% with 1 mg/L collagen and by 21% with 12.5 mg/L collagen (Table 3) (Keevil 2000). The different order of magnitude observed with the two concentrations of the same agonist is worth noting. However, no significant effect on ADP-or thrombin-induced platelet aggregation was observed, indicating that only the collagen-induced signaling pathway was modulated by the intervention (Keevil 2000), which is in contrast to the observations of van Mierlo (2010), who reported a trend for inhibited ADP-induced platelet aggregation, but no changes when collagen was used as agonist. Both studies were conducted on healthy volunteers with grape-derived intervention products, but different methods were applied to measure platelet function (Table 3). The study of Keevil (2000) was uncontrolled and had an acute-on-chronic design, which might have contributed to discrepant findings. This area merits further investigation in well-designed RCTs, especially given the

promising anti-platelet effects observed with flavan-3-ols and isoflavones (Bachmair 2014, Ostertag 2010).

1.3.2.3 Effects of ACNs on circulating EMPs and PMPs

To our knowledge, there are no intervention studies that have investigated the acute effect of ACN-rich interventions on the number of circulating PMPs or EMPs, and there is only one intervention study that investigated the effect of other flavonoid-rich interventions (Table 3). In this study, consumption of a polyphenol-rich cocoa beverage by healthy men and women (n = 30) significantly reduced the number of circulating EMPs at 2 and 6 h post-consumption compared to baseline (Rein 2000), but the number of circulating PMPs was not assessed. These promising results warrant more research in this area

1.3.2.4 Effects of ACNs on markers of inflammation

The evidence on the effect of berry consumption on markers of inflammation has recently been reviewed. Joseph (2014) identified three studies investigating the acute effects of ACN-rich berries in response to meals on selected markers of inflammation. There was no effect on intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 following consumption of a blackcurrant beverage providing a low dose of ACNs (51 mg) in healthy volunteers (Jin 2011), but no other markers were measured in this study. Consumption of strawberry beverages containing 94 mg total polyphenols together with meals was reported to attenuate the postprandial inflammatory response (IL-6, IL-1β, CRP) in overweight volunteers (Edirisinghe 2011, Ellis 2011), suggesting that ACNs can have short-term beneficial effects on inflammatory responses.

1.3.3 Chronic human intervention trials on ACNs and selected markers of CVD risk

This chapter is confined to human intervention studies with vascular function, platelet function, BP, arterial stiffness, MPs and inflammatory marker endpoints.

1.3.3.1 Effects of ACNs on vascular function, arterial stiffness and blood pressure

A summary of the studies is presented in Table 4. The chronic effect of ACN-rich interventions on vascular function was investigated in five studies, with the majority reporting favorable outcomes. As such, improvements in FMD were reported after consumption of 320 mg of purified ACNs for 12 weeks in hypercholesterolemia subjects (Zhu 2011), after consumption of a blueberry smoothie for 6 weeks in volunteers with metabolic syndrome (Stull 2015), after consumption of boysenberry beverage (351 mg TP) for 28 days in a small open-label, uncontrolled study on 6 volunteers (Matsusima 2013) and after consumption of a blackcurrant juice drink (815 mg TP) for 6 weeks in 66 healthy volunteers (Khan 2014). Interestingly, Khan's study (2014) indicates that dose might be important as no significant improvement was observed with a lower dose of the blackcurrant juice drink. However, unfortunately, the publication does not provide the daily polyphenol dose of the low dose blackcurrant juice drink and so it is difficult to draw conclusions based on this. Similarly, no effect on EndoPAT vascular function was observed after 6-week consumption of blueberry smoothie in volunteers at increased CVD risk (Riso 2013).

Regarding BP, as summarized in Table 4, most studies reported no chronic effect of ACN-rich interventions consumed for 3-12 weeks on BP. This is in line with results from a recent Prisma-compliant meta-analysis of 6 RCTs concerned with the effect of 4-12 week ACN supplementation on BP, which concluded that there was no significant effect on either SBP or

DBP after 4-12 week ACN supplementation, but recognizes that additional well-designed RCTs with large volunteer numbers and long follow-up periods are required (Zhu 2016). A reduction in BP was only reported in 4 out of 14 studies reviewed in the present thesis, but there is some indication that the SBP at baseline might be an important determining factor. In the majority of studies that did not report a BP effect, the average baseline SBP was <130 mm Hg, whereas it was ≥130 mm Hg in studies that did show an effect. This argument is further supported by an 8-week intervention study with mixed berries where a subgroup analysis of the BP data revealed that the SBP-lowering effect was only significant in volunteers with the highest tertile of baseline SBP (133-185 mm Hg) (Erlund 2008). This observation warrants confirmation in subsequent studies, especially given the clinically relevant reduction in BP observed in subjects with elevated BP. For example, there was a significant reduction in BP after 8 weeks of twice-daily blueberry consumption: SBP decreased by 7 mm Hg (from 138 to 131 mm Hg, $P<0.05$) and DBP decreased by 5 mm Hg (from 80 to 75 mm Hg, $P<0.01$), while no significant changes were recorded in the placebo group. Interestingly, no significant change was recorded at the 4 week measurement (Johnson 2015), indicating that a long-term intervention is required for BP-lowering effect. A similar magnitude of reduction was observed in an 8-week intervention study with a blueberry beverage, where SBP decreased by 7.8 mm Hg ($P<0.005$) and DBP decreased by 2.5 mm Hg ($P<0.05$), but unfortunately there is no information provided regarding the baseline BP in order to draw further conclusions from this data (Basu 2010a).

Table 4 Human intervention studies investigating the chronic effects of ACN-rich interventions on vascular function, arterial stiffness, blood pressure, platelet function and circulating EMPs and PMPs

Outcome	Intervention	Polyphenol dose (mg) / day	Subjects	Study design	Intervention period	Results	Reference
Vascular function, arterial stiffness and blood pressure	Blueberries	Not measured	n = 20, chronic smokers, mean age: 28 y mean BP: 128/84 mm Hg (approx., read from graph)	RCT, parallel	B, 3 wk	↔ BP ↔ ACE activity	McAnulty (2005)
	Chokeberry capsules	64 ACN, 128 Pro, 23 PA	n = 44, M+F, coronary artery disease and statins, mean age: 66 y mean BP: 130/84 mm Hg	RCT, DB, parallel	B, 6 wk	↓ SBP & DPB ↓ ACE activity (only tested in a subgroup previously untreated with ACE)	Naruszewicz (2007)
	"Mixed berries"[1]	837 TP, 515 ACN, 240 Pro, 63 PA, 62 flavanones	n = 72, M+F, at least one CVD risk factors, mean age: 58 y mean BP: 130/81 mm Hg	RCT, SB, parallel	B, 8 wk	↓ SBP (subgroup analysis revealed that reduction was only significant in volunteers with the highest tertile of B SBP (133-185 mm Hg)	Erlund (2008)
	Elderberry extract capsules	500 ACN (Cy glycosides)	n = 52 F (postmenopausal), healthy, mean age: 58 y, mean BP: 127/80 mm Hg	RCT, DB, parallel	B, 12 wk	↔ BP	Curtis (2009)
	Freeze-dried blueberry beverage	1,624 TP, 742 ACN	n = 48, M+F, obese, mean age: 50 y B BP not provided	RCT, SB, parallel	B, 8 wk	↓ SBP & DPB	Basu (2010a)
	Freeze-dried strawberry beverage	2,006 TP, 154 ACN	n = 27, M+F, obese with metabolic syndrome, mean age: 47 y mean BP: 133/83 mm Hg	RCT, parallel	B, 8 wk	↔ BP	Basu (2010b)
	Purified bilberry and blackcurrant ACN capsules	320mg ACN: 59% Dp glycosides, 33% Cy glycosides	n = 146, M+F, hypercholesteremic, age: 40-65 y, mean BP: 125/84 mm Hg	RCT, DB, parallel	B, 12 wk	↑ FMD ↑ cGMP ↔ BP	Zhu (2011)

[1] volunteers consumed two berry portions per day from four products: whole bilberries, lingonberry nectar, puree of blackcurrants and strawberries, chokeberry and raspberry juice

Outcome	Intervention	Polyphenol dose (mg) / day	Subjects	Study design	Intervention period	Results	Reference
Vascular function, arterial stiffness and blood pressure	Purified bilberry and blackcurrant ACN capsules	640mg ACN (mainly Dp and Cy glycosides)	n = 27, M, mild hypertension (BP: 143/96 mm Hg at screening), not on antihypertensive or lipid-lowering medication age: 35-51 y,	RCT, DB, crossover	B, 4wk	⇔ BP (sitting, supine and 24 h ambulatory)	Hassellund (2012)
	Boysenberry beverage	351 TP, (4.65 flavanols)	n = 6, M+F, mean age: 34-68 y, SBP: 120-172 mm Hg	open-label, uncontrolled	B, 14, 28 d	↑ FMD at 14 & 28 d ⇔ BP	Matsusima (2013)
	Blueberry beverage	375 ACN, 128 CA	n = 18, M, at least one CVD risk factors, mean age: 48 y, mean baseline BP: 121/79 mm Hg	RCT, crossover	B, 6 wk	⇔ EndoPAT ⇔ BP	Riso (2013)
	Freeze-dried strawberry beverage	1,001-2,005 TP, 78-155 ACN, 106-220 EA, 23-50 phytosterols	n = 60, M+F, abdominal adiposity & dyslipidemia, mean BP: 132/86 mm Hg, mean age: 49 y mean BP: 132/86 mm Hg	RCT, parallel 4-arm	B, 12wk	⇔ BP	Basu (2014)
	Blackcurrant juice drink, 2 arms: - low dose - high dose	**low dose:** not provided **high dose:** 815 TP	n = 66, M+F, healthy, mean age: 53 y, mean BP: 128/81 mm Hg	RCT, DB, parallel	B, 6 wk	**low dose:** ⇔ FMD ⇔ BP **high dose:** ↑ FMD ⇔ BP	Khan (2014)
	Blueberry beverage	845 TP, 470 ACN	n = 48, F (postmenopausal), mean age: 58 y, mean BP: 138/79 mm Hg (125/85-160/90 mm Hg)	RCT, DB, parallel	B, 8 wk	↓ SBP and DPB ↓ baPWV ⇔ cfPWV ↑ NO	Johnson (2015)
	Blueberry smoothie (containing yoghurt & milk)	1,547 TP, 581 ACN	n = 44, M+F, metabolic syndrome, mean BP: 125/80 mm Hg, most on antihypertensive medication mean age: 57 y mean baseline BP: 125/80 mm Hg	RCT, DB, parallel	B, 6 wk	↑ EndoPAT ⇔ BP	Stull (2015)

Outcome	Intervention	Polyphenol dose (mg) / day	Subjects	Study design	Intervention period	Results	Reference
Platelet function	Purple grape juice ("Welch's Concord") (7 mL/kg/d)	Not determined, but main classes were cinnamic acid, ACNs, flavonols and Pro	n = 20, M+F, healthy, mean age: 31 y	uncontrolled	B, 2 wk	↓ platelet aggregation induced by PMA (58 to 39%), ADP (18%↓) and collagen (13%↓) ↑ platelet-derived NO production ↓ superoxide release	Freedman (2001)[2]
	Purple grape juice ("Welch's Concord") (7 mL/kg/d)	Not determined	n = 20 M+F, stable coronary artery disease, all taking aspirin and some additionally on further standard medication, mean age: 63 y	RCT, DB, crossover	B, 2 wk	⇔ platelet aggregation ⇔ platelet-derived NO production ⇔ plasma TXB_2 levels and P-selectin ↓ platelet-derived superoxide production ↓ CD40L	Albers (2004)[3]
	"Mixed berries"[4]	837 TP, 515 ACN, 240 Pro, 63 PA, 62 flavanones	n = 72, M+F, at least one CVD risk factors, mean age: 58 y	RCT, SB, parallel	B, 8 wk	↓ closure time ⇔ Plasma biomarkers of platelet activation, coagulation and fibrinolysis	Erlund (2008)[5]
	Elderberry extract capsules	500 ACN (mainly Cy-3-glc)	n = 52, F (postmenopausal), healthy, mean age: 58 y	RCT, DB, parallel	B, 12 wk	⇔ platelet activation	Curtis (2009)[6]
	Strawberry beverage vs placebo	95 TP, 39 ACN	n = 24, M+F, overweight, mean age: 50 y	RCT, SB, unclear whether crossover or parallel[7]	B, 6 wk, (But also 6 h postprandially)	⇔ closure time	Ellis (2011)[8]
	Aronia melanocarpa extract	60 TP, 29 ACN	n = 38, M+F, metabolic syndrome, age: 42-65 y	uncontrolled	B, 1, 2 mo	↓ platelet aggregation at 1 mo ⇔ platelet aggregation at 2 mo	Sikora (2012)[9]

[2] method for platelet function measurement: impedance aggregometry in whole blood (activators: PMA, ADP & collagen)
[3] method for platelet function measurement: light transmission aggregometry in washed platelets (activators: ADP (5 µmol/L), TRAP and PMA (concentration not provided)), platelet-derived NO production, platelet-derived superoxide production, markers of platelet activation: TXB_2, P-selectin, CD40L
[4] volunteers consumed two berry portions per day from four products: whole bilberries, lingonberry nectar, puree of blackcurrants and strawberries, chokeberry and raspberry juice
[5] method for platelet function measurement: platelet function analyzer 100 (activators: ADP/collagen)
[6] method for platelet function measurement: platelet activation using whole blood flow cytometry: P-selectin and the activated fibrinogen receptor GPIIb/IIIa (activator: ADP, collagen and non-stimulated platelets)
[7] whilst the abstract describes a "crossover design", the experimental section describes a "6-wk parallel-design dietary intervention trial"
[8] method for platelet function measurement: platelet function analyzer 100 (activators: epinephrine/collagen and ADP/collagen)
[9] method for platelet function measurement: light transmission aggregometry in PRP (activator: ADP (10 µmol/L))

Outcome	Intervention	Polyphenol dose (mg) / day	Subjects	Study design	Intervention period	Results	Reference
Platelet function	Strawberries	1,130 TP, 253 ACN	n = 10, M+F, healthy, mean age: 27 y	uncontrolled	B, 1 mo	↓ percentage of activated platelets after 30 d compared to baseline ⇔ percentage of platelets in the post activation phase	Alvarez-Suarez (2014)[10]
	2 Grape extract capsules: grape-red wine and grape alone	Grape-red wine[11] / grape alone: 800 / 800 TP, 21.5 / 225.8 ACN, 45.2 / 0.4 catechins, 4.8 / 9.5 flavonones, 7.9 / 5.2 PA	n = 60, M, mildly hypertensive, mean age: 59 y	RCT, DB, incomplete crossover[12]	B, 4 wk	⇔ closure time with both intervention products	Draijer (2015)[13]
Circulating EMPs and PMPs	Cocoa beverage vs macro-and micronutrient matched control	750 TP (control: 18 TP)	n = 16, M+F, coronary artery disease, on standard medication, mean age: 64 y	RCT, DB, crossover	B, 30 d	↓ EMPs ⇔ PMPs EMPs inversely correlated with FMD (intervention also improved FMD)	Horn (2014)[14]
	Natural cocoa-bar vs placebo	702 TP, 640 Pro, 48 Epicatechin, 14 Catechin	n = 24, F, healthy, 3 BMI groups: normal, overweight and obese, mean age: 22 y	RCT, DB, crossover	B, 4 wk	↓ EMPs in whole group **Subgroup analysis:** ↓ EMPs in obese volunteers ↓ EMPs in overweight volunteers ⇔ EMPs in normal weight volunteers	McFarlin (2015)[15]

ACE, angiotensin converting enzyme; ACN, anthocyanins; B, baseline; BP, blood pressure; CA, chlorogenic acid; CD40L, CD40 ligand; cGMP, cyclic guanosine monophosphate; CVD, cardiovascular disease; Cy, cyanidin; Cy-3-glc, cyanidin-3-O-glucoside; DB, double-blind; DBP, diastolic blood pressure; Dp, delphinidin; EA, Ellagic acid; EMP, endothelial-derived microparticles; FMD, flow-mediated dilation; NO, nitric oxide; PA, phenolic acids; PMA, phorbol 12-myristate 13-acetate; PMP, platelet-derived microparticles; Pro, procyanidins; PWV, pulse wave velocity; RCT, randomized controlled trial; SB, single-blind; SBP, systolic blood pressure; TP, total polyphenols; TRAP, thrombin receptor activating peptide; TXB_2, thromboxane B2.

[10] method for platelet function measurement: platelet activation assessed microscopically in non-stimulated platelets in PRP and classified according to ultrastructure as: unstimulated, activated, post activation phase

[11] for both products the majority of phenolics remained unidentified (but are believed to be mainly polymeric proanthocyanidins)

[12] 30 volunteers had placebo & grape-red wine, whilst 30 volunteers had placebo & grape alone

[13] method for platelet function measurement: platelet function analyzer 100; activators: epinephrine/collagen and ADP/collagen

[14] method for EMP/PMP measurement: Flow cytometry, PMP: $CD41^+$, EMP: $CD144^+$ or $CD31^+/CD41^-$

[15] method for EMP measurement: Flow cytometry: EMP: CD42a−/45−/144+

1.3.3.2 Effects of ACNs on platelet function

Few studies have investigated the chronic effects of ACN-rich interventions on platelet function (Table 4), with several different methodologies being applied to assess platelet function.

The platelet function analyzer was used in three studies. No modulation in platelet function was observed after 6 weeks of consuming a strawberry beverage containing 95 mg TP by 24 middle-aged, overweight volunteers (Ellis 2011) or after 4 week consumption of grape extract capsules (800 mg TP, 226 mg ACNs) by 60 mildly hypertensive men in a double-blind RCT (Draijer 2015). In contrast, a significant inhibition in ADP/collagen-induced closure time was demonstrated in a longer study (8 weeks) with more volunteers (n = 72) and a higher daily ACN dose (515 mg, 837 mg TP). This study was conducted on middle-aged subjects with increased CVD risk and investigated the effect of daily consumption of a combination of various berries (whole bilberries, lingonberry nectar, puree of blackcurrants and strawberries, chokeberry and raspberry juice). Berry consumption increased polyphenol concentrations in plasma and prolonged occlusion time by 11%. However, as this study applied a whole-food approach, the intervention products provided not only various polyphenols, but also other potentially bioactive components (such as 62 mg vitamin C and 237 mg potassium per day), that were not controlled for. Thus, whilst this study provides promising results, it does not allow elucidation of compounds responsible for the observed effect and it remains possible that the intervention contained non-phenolic compounds affecting platelet aggregation (Erlund 2008).

Platelet activation, as assessed by quantification of platelet surface receptors using whole blood flow cytometry, was measured in two studies. There were no significant effects on plasma biomarkers of platelet activation, coagulation and fibrinolysis in the study of Erlund (2008). Similarly, no significant effect on platelet activation was demonstrated in 52 healthy

postmenopausal women consuming elderberry extract capsules 2x/d (containing 500 mg ACNs in the form of Cy-3-glc) (Curtis 2009).

Platelet activation was assessed microscopically in non-stimulated platelets in one study. Most platelets remained in the unstimulated state (99.05% of platelets at baseline vs 99.34% of platelets post-supplementation) upon consumption of a high dose of daily strawberries for 1 month containing 1,130 mg TPs and 253 mg ACNs and there was no significant change in the percentage of platelets in the post activation phase. There was, however, a statistically significant decrease in the percentage of activated platelets, albeit of a very small magnitude (0.29% of platelets at baseline vs 0.09% of platelets post-supplementation). The clinical relevance of such a small decrease is unknown (Alvarez-Suarez 2014).

The extent of platelet aggregation following *ex vivo* agonist stimulation was measured in three studies with mixed results. A significant inhibition in *ex vivo* ADP-induced platelet aggregation was demonstrated in an uncontrolled pilot study upon daily ingestion of 100 mg aronia melanocarpa containing 60 mg TPs and 29 mg ACNs in 38 middle-aged participants with metabolic syndrome, however, the effect was not statistically significant after 2 months of supplementation (Sikora 2012). In another study, a significant reduction in phorbol 12-myristate 13-acetate (PMA)-, ADP- and collagen-induced platelet aggregation was demonstrated after 14 days intervention with purple grape juice (7 mL/kg/d) in 20 healthy male and female volunteers (Freedman 2001). The discrepant findings between the two studies could have arisen by different techniques to measure platelet function, differences in the population group studied (healthy vs mildly hypertensive) or differences in intervention used (the purple grape juice in Freedman's study was not very well characterized and the polyphenol dose not provided). Some valuable insight into the mechanism of action was also provided in the study of Freedman (2001): The platelet-inhibitory effect was accompanied by a decrease in platelet-derived superoxide anion production (which enhances platelet aggregation) and platelet-

derived NO production and these effects were shown to be dose-dependent. A further study by the same research group indicates that differences in health and/or medication status may account, at least in part, for discrepant findings. In contrast to the evidence obtained in healthy volunteers (Freedman 2001), there was no statistically significant anti-platelet effect when the same intervention was administered to volunteers with stable coronary artery disease taking aspirin. Whilst the discrepant findings could have arisen due to aspirin usage in one study, it is also possible that difference in health status made a contribution (Albers 2004).

In conclusion, the number of studies in this area is limited and comparison only possible to a limited extent due to differences in the intervention type (grape vs mixed berries vs elderberry vs strawberry vs aronia), polyphenol/ACN dose (TP range: 60-1,130 mg, ACN range: 21.2-515 mg), intervention length (range: 2-12 weeks), population group characteristics and platelet function assessment methods. An additional limitation is that some of the studies were only single-blind and/or were lacking a control group. These factors may have contributed to the contradictory results, with some studies suggesting platelet-inhibitory properties, and others indicating no effect. As such, this area warrants further research before a firm conclusion can be reached. It is also worth noting in this context that more research exists with other flavonoid classes, particularly the flavan-3-ols and isoflavones. Strong evidence for an anti-platelet effect of these compounds has been provided by a recent review of human RCTs investigating the effect of flavan-3-ol-rich cocoa (products) and grape seed extract on platelet function, and the evidence was particularly convincing after acute consumption (Bachmair 2014). Similarly, a review of purified isoflavones provides evidence for platelet-inhibitory effects (Nardini 2007).

1.3.3.3 Effects of ACNs on circulating EMPs and PMPs

To our knowledge, there are no intervention studies that have investigated the chronic effect of ACN-rich interventions on the number of circulating PMPs or EMPs, and only two human intervention studies investigated the effects of other flavonoid-rich interventions (Table 4).

A reduction in the number of circulating EMPs was demonstrated in a crossover RCT upon consumption of cocoa polyphenols for 30 days in patients with coronary artery disease and this effect was paralleled by improvements in vascular function (assessed by FMD), suggesting a possible causal link (Table 4) (Horn 2014). There was, however, no effect on the number of circulating PMPs. Similarly, reduced levels of circulating EMPs were observed after daily consumption of a natural cocoa bar by young, healthy women (Table 4) (McFarlin 2015). Interestingly, a subgroup analysis revealed that this effect was only significant in obese and overweight volunteers who had higher initial values, but not in volunteers with a normal body weight (Table 4) (McFarlin 2015), indicating that interventions might be particularly effective in volunteers at risk of CVD with elevated MPs levels, but the number of people in each subgroup was small (≤10) and this needs to be confirmed in further studies before a firm conclusion can be reached. The beneficial effect of polyphenol-rich interventions on MPs was also confirmed in a rodent model. In rats with aldosterone-induced hypertension, daily consumption of red wine polyphenols for 4 weeks significantly reduced the number of circulating EMPs and PMPs (Andres 2012).

1.3.3.4 Effects of ACNs on markers of inflammation

The evidence on the effect of chronic ACN consumption on markers of inflammation has recently been reviewed. Mena (2014) identified 15 chronic studies with a study duration between 2 and 24 weeks. The authors report the findings to be very inconsistent, and ascribe the inconsistencies to differences in the type of population studied (healthy vs overweight vs

obese vs hypercholesterolemic vs prehypertensive vs volunteers with metabolic syndrome), length of study period (2-24 weeks), source of ACNs (tart cherry vs purified ACNs vs red grapes vs bilberries vs purple carrot vs blueberry), and daily dose provided (45-1323 mg ACNs/d). Nevertheless, it was observed that ACNs have no effects in studies of shorter duration (2-6 weeks) and in relatively young population groups (23-50 years). From a limited number of studies with a longer duration, the author speculate that lipid modulation might be the underlying mechanism of action for chronic anti-inflammatory effects of ACNs (Mena 2014).

It is further notable that the majority of studies included inflammatory mediators as secondary outcomes. As such, most studies were based on sample sizes which were probably too small to yield robust results, given the high standard deviation for most inflammatory markers. The same shortcoming has recently been observed in studies investigating the effects of long-chain n-3 polyunsaturated fatty acids. An RCT overcoming this limitation and specifically investigating the effects of EPA and DHA on inflammatory markers as primary outcome measure, estimated a required sample size of 150 volunteers to detect a 10% difference in plasma CRP levels (Allaire 2016). In contrast, only two out of the 15 studies reviewed by Mena (2014) included more than 100 volunteers.

1.3.3.5 Summary of human study evidence

In summary, the literature review demonstrated acute benefits of ACN-rich interventions on vascular function in five out of eight studies, but most studies showing a beneficial effect were conducted with blueberry interventions, which are not only rich in ACNs but also proanthocyanidins and chlorogenic acid, and data on other ACN sources are limited.

Apart from modulation of vascular function, there are other potential mechanisms by which ACNs may reduce the risk of CVD, including modulation of platelet activation and aggregation,

modulation of MP release, modulation of cytokine production and modulation of arterial stiffness.

A recent review of human RCTs concludes that there is strong evidence for an anti-platelet effect of flavan-3-ol-rich cocoa (products) and grape seed extract, particularly after acute consumption (Bachmair 2014), but little is known about the effect of ACN-rich interventions.

Emerging evidence suggests that consumption of cocoa polyphenols reduces the number of circulating EMPs in patients with coronary artery disease (Horn 2014) as well as obese and overweight women, while no effects were observed in normal weight women (McFarlin 2015), indicating that interventions might be particularly effective in volunteers at risk of CVD with elevated MP levels, but the number of people in each BMI subgroup was small (≤10) and this needs to be confirmed in further studies before a firm conclusion can be reached. To our knowledge, no RCTs have investigated the effect of ACNs on the number of circulating MP.

In conclusion, further human RCTs are needed to corroborate previously reported beneficial effects of dietary ACNs on vascular function and investigate potential benefits on largely unexplored markers of CVD risk (MPs and platelet function).

1.3.4 Cell culture experiments on ACNs and cytokines

A number of cell culture studies have investigated the ability of ACNs to modulate cytokine production. However, the majority were conducted using the unmetabolized parent ACNs rather than the physiologically more relevant metabolites and often testing supraphysiologically high concentrations (Wang and Mazza 2002, Herath 2003, Jin 2006, Bognar 2013, Esposito 2014, Zhang 2010, Karlsen 2007, Karlsen 2010, Roth 2014, Mossalayi 2014, Del Corno 2014, Zdarilova 2010, Noratto 2011). Only a limited number of studies have employed physiologically relevant compounds and doses (Table 5). In one of those studies, a number of

Cy-3-glc metabolites were shown to reduce IL-6 secretion by stimulated human vascular endothelial cells (HUVECs) and the effect was paralleled by reduced mRNA expression (Amin 2015). Interestingly, the metabolites were reported to be more potent than the parent compound. Similarly, several microbial phenolic metabolites reduced lipopolysaccharide (LPS)-stimulated IL-6 secretion by human peripheral blood mononuclear cells (Monagas 2009). In addition, a number of flavonoid metabolites were shown to reduce TNF-α production by LPS-induced murine 264.7 macrophages (Min 2010) and THP-1 monocytes (di Gesso 2015). The latter study also tested combinations of phenolic compounds and the data are indicative of additive/synergistic effects. IL-1β production was reduced by PCA in LPS-stimulated murine macrophages (Min 2010) and by 4-HBA in LPS-stimulated THP-1 monocytes, but a number of other flavonoid metabolites had no effect (di Gesso 2015). This study also investigated the effects of a number of flavonoid metabolites on IL-10 production, but reports no significant effects (di Gesso 2015). Furthermore, studies concerned with the bioactivity on IL-8 are lacking. Research is also lacking on the effect of ACNs and their metabolites on the potential modulation of cytokine production by human-derived macrophages, as are studies comparing the effects in human-derived monocytes vs macrophages.

Table 5 Effects of ACNs and their metabolites on cytokine production in cell culture models

Test compounds	Cell model	Changes in cytokine production	Reference
3,4-DHPPA 3-hydroxyphenylpropionic acid 3,4-DHPAA 3-hydroxyphenylacetic acid 4-HBA 4-HHA *(All at 1μM)*	Human peripheral blood mononuclear cells (n=6) stimulated with LPS	**↓ TNF-α protein:** 3,4-DHPPA 3,4-DHPAA 4-HHA **↑ TNF-α protein:** 4-HBA **↓ IL-1β protein:** 3,4-DHPPA 3,4-DHPAA **↓ IL-6 protein:** 3,4-DHPPA 3,4-DHPAA	Monagas 2009
Cy-3-glc Cy PCA *(All at 1, 2 & 5μM)*	Murine macrophages (RAW 264.7) stimulated with LPS	**↓ TNF-α protein:** Cy-3-glc (2 & 5μM) Cy (1, 2 & 5μM) PCA (1, 2 & 5μM) **↓ IL-1β protein:** Cy-3-glc (1, 2 & 5μM) Cy (1, 2 & 5μM) PCA (1, 2 & 5μM)	Min 2010
quercetin naringenin epicatechin hesperidin Pn-3-glc Cy-3-glc 4-HBA PCA IVA VA BA-gluc BA-sulfate PCA-3-gluc PCA-4-gluc PCA-3-sulfate PCA-4-sulfate IVA-gluc IVA-sulfate VA-glucuronide VA-sulfate 29 combinations *(All at 0.1, 1, 10 and 100μM)*	Human monocytes (THP-1) stimulated with LPS	**↓ TNF-α protein:** Cy-3-glc (1μM) Pn-3-glc (0.1, 1 & 10μM) BA-sulfate (1μM) IVA (1μM) IVA-gluc (0.1, 1 & 10μM) VA-gluc (1 & 10μM) PCA-3-sulfate (1 & 10μM) Combination: PCA+4-HBA (0.1, 1 & 10μM) Combination: PCA+VA+4-HBA (1 & 10μM) Combination: 4-HBA+BA-gluc+BA-sulfate (10μM) Combination: PCA+PCA-3-gluc (1 & 10μM) **↓ IL-1β protein:** 4-HBA (1μM)	Di Gesso 2015
Cy-3-glc PCA PGA VA IVA PCA-3-gluc PCA-4 gluc PCA-3-sulfate PCA-4-sulfate VA-4-sulfate IVA-3-sulfate FA *(All at 0.1, 1 and 10μM)*	Human vascular endothelial cells (HUVEC) stimulated with oxidized LDL or CD40L	**Oxidized LDL stimulation:** **↓ IL-6 protein:** PCA (0.1, 1 & 10μM) VA (0.1, 1 & 10μM) PCA-3-gluc (0.1, 1 & 10μM) PCA-4-gluc (0.1, 1 & 10μM) PCA-3-sulfate (0.1, 1 & 10μM) PCA-4-sulfate (0.1, 1 & 10μM) VA-4-sulfate (0.1, 1 & 10μM) IVA-3-sulfate (0.1, 1 & 10μM) FA (0.1, 1 & 10μM) **CD40L stimulation:** **↓ IL-6 protein:** Cy-3-glc (0.1 & 1μM) PCA (1 & 10μM) VA (0.1, 1 & 10μM) IVA (0.1, 1 & 10μM) PCA-3-gluc (0.1 & 1μM) PCA-3-sulfate (10μM) PCA-4-sulfate (0.1, 1 & 10μM) IVA-3-sulfate (0.1 & 1μM)	Amin 2015

3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3,4-DHPPA, 3,4-dihydroxyphenylpropionic acid; 4-HBA, 4-hydroxybenzoic acid; 4-HHA, 4-hydroxyhippuric acid; BA, benzoic acid; CD40L, CD40 ligand; Cy, cyanidin; Cy-3-glc, cyanidin-3-O-glucoside; FA, ferulic acid; gluc, glucuronide; IL, interleukin; IVA, isovanillic acid; LDL, low density lipoprotein; LPS, lipopolysaccharide; PCA, protocatechuic acid; PGA, phloroglucinaldehyde; Pn-3-glc, peonidin-3-O-glucoside; TNF-α, tumor necrosis factor-α; VA, vanillic acid.

1.4 Structure and development of the PhD project in collaboration with GlaxoSmithKline

GlaxoSmithKline supported this PhD project as part of a BBSRC CASE studentship and the original brief was to examine potential effects of any berry-derived ACNs and their metabolites on immunity and inflammation. Chapter 2 and 3 of the present PhD thesis were conducted on this basis and it was envisaged that this leads to a clinical study that would constitute Chapter 4. However, evolution of GlaxoSmithKline's corporate structure and a number of changes in industrial supervisors lead to diminished interest in immune and inflammation work. In addition, power calculations suggested that the size of a clinical immunity/inflammation study would need to be unfeasibly large. Furthermore, there was a lack of credible published evidence in this area and the experiments presented in Chapter 2 and 3 of this thesis had shown little effect of anthocyanins on innate immunity, i.e. did not support the extension to an *in vivo* study. Since there was much stronger evidence for effects of flavonoids on vascular function and a clear knowledge gap relating to anthocyanins in this context, we believed that a study examining the effects of a super-enriched anthocyanin beverage on vascular function and associated inflammation would be extremely important and timely, and it was decided to conduct a clinical study in this area. Consequently, the PhD thesis is presented as stand-alone chapters.

1.5 Aims and objectives of the thesis

The overall aim of this PhD research project was to evaluate the ability of ACNs to improve markers of immunity and CVD risk.

Specific PhD objectives:

1. To characterize the *in vitro* effects of the strawberry-derived ACN Pg-3-glc and its physiologically relevant metabolites on the production of selected pro- and anti-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated THP-1 cells and to compare the effects in human-derived monocytes vs macrophages (Chapter 2).

2. To characterize the *ex vivo* effects of the strawberry-derived ACN Pg-3-glc and its physiologically relevant metabolites on phagocytosis, oxidative burst and LPS-stimulated cytokine production in whole blood cultures (Chapter 3).

3. To characterize the *in vivo* acute effects of an ACN-rich blackcurrant beverage on FMD, platelet aggregation and a range of further CVD risk markers in response to a high-fat meal in a healthy, middle-aged population (Chapter 4).

1.6 Study hypothesis

It is hypothesized that ACNs, either in purified form or delivered within a blackcurrant beverage, improve selected markers of immunity and CVD risk and that the beneficial effect will be seen *in vitro*, *ex vivo* and *in vivo*.

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Chapter 2 Effects of a strawberry-derived anthocyanin and its metabolites on lipopolysaccharide-stimulated cytokine production by THP-1 monocytes and macrophages

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Abstract

Epidemiological evidence suggests cardioprotective effects of anthocyanin consumption. This study tested the anti-inflammatory effects of the predominant strawberry anthocyanin, pelargonidin-3-O-glucoside (Pg-3-glc), and three of its plasma metabolites (4-hydroxybenzoic acid, protocatechuic acid [PCA], phloroglucinaldehyde [PGA]). THP-1 monocytes and macrophages were stimulated with lipopolysaccharide in the presence of Pg-3-glc or three of its plasma metabolites at concentrations of 0 to 20 μM. Concentrations of selected cytokines (tumor necrosis factor-α [TNF-α], interleukin [IL]-1β, IL-6, IL-8 and IL-10) were determined using a cytometric bead array kit. PCA at 0.31, 1.25 and 20 μM and PGA at 5 and 20 μM decreased the concentration of IL-6 in the monocyte cultures, but there were no effects on TNF-α, IL-1β, IL-8 and IL-10 and there were no effects of the other compounds. In the macrophage cultures, PGA at 20 μM decreased the concentrations of IL-6 and IL-10, but there was no effect on TNF-α, IL-1β and IL-8 and there were no effects of the other compounds. In conclusion, while the effects of PGA were only observed at the higher, supraphysiological concentration and are thus considered of limited physiological relevance overall, the anti-inflammatory properties of PCA were observed at both the lower, physiologically relevant, and the higher concentrations; however, effects were modest and limited to IL-6 and monocytes. These preliminary data suggest potential for physiologically attainable PCA concentrations to modulate IL-6 production by monocytes.

Keywords: Anthocyanin, Cytokine, Inflammation, Pelargonidin-3-O-glucoside, Strawberry

2.1 Introduction

Cardiovascular disease (CVD) remains a major cause of morbidity and mortality throughout the world [1]. Strawberries constitute a popular fruit and they are particularly rich in anthocyanins [2]. Recently, anthocyanins have gained interest as potential cardioprotective compounds as epidemiological evidence has linked their consumption with a lower risk of CVD [3-5] and CVD risk factors [6-8]. The mechanisms by which anthocyanins mediate the cardioprotective effects are not yet fully elucidated, but evidence suggests modulation of vascular function, platelet aggregation and inflammation [9-15]. TNF-α, IL-1β, IL-6, IL-8 and IL-10 are inflammatory cytokines that play a critical role in atherosclerosis, the underlying cause of most CVDs [16]. Anthocyanins and their degradants have been demonstrated to possess a number of anti-inflammatory actions. Experiments employing cell culture models have demonstrated inhibitory action of anthocyanin-rich extracts, parent anthocyanins and their metabolites on the gene expression and secretion of pro-inflammatory adhesion molecules [9, 13, 17-20] and inhibitory effects on the expression of pro-inflammatory enzymes, such as cyclooxygenase-2 [11, 21, 22] and inducible nitric oxide synthase [11, 21, 22] and, consequently, a reduction in the generation of inflammatory mediators, such as nitric oxide [11, 22] and prostaglandin E2 [11, 22]. As such, it appears that anthocyanins may confer anti-inflammatory actions via several routes and there is evidence to suggest that modulation of cytokine production might also be involved [9-13, 18, 19, 21, 23-31].

Pelargonidin glycosides are the predominant anthocyanins in strawberries. Glucuronidated pelargonidin has been reported as the predominant metabolite in three pharmacokinetic studies [32-34], but there is ambiguity regarding the position of glucuronidation and glucuronidated pelargonidin compounds are currently commercially unavailable and hence cannot be tested in cell culture models. 4-hydroxybenzoic acid (4-HBA) and protocatechuic acid (PCA) have also been reported in plasma following strawberry consumption in low micromolar concentrations

(0.1-2 μM) [34-36]. In addition, it is likely that phloroglucinaldehyde (PGA) might appear in plasma following strawberry consumption, as it is a A-ring degradant, reported in plasma upon anthocyanin consumption in low to high nanomolar concentrations (20-600 nM) [37, 38]. However, most studies exploring the effect of anthocyanins to modulate cytokine secretion were conducted using the unmetabolized parent anthocyanins rather than the physiologically more relevant metabolites and often testing supraphysiologically high concentrations [9, 10, 19, 21, 23-29, 39, 40]. In addition, macrophages are an important source of inflammatory cytokines [41], but to our knowledge there are no studies exploring the potential modulation of cytokine production by anthocyanins or their metabolites by human-derived macrophages, or studies comparing the effects in human-derived monocytes vs macrophages. To address these knowledge gaps, the aim of this study was to compare the effect of the parent anthocyanin pelargonidin-3-O-glucoside (Pg-3-glc) and three physiologically relevant plasma metabolites on the production of selected pro- and anti-inflammatory cytokines (tumor necrosis factor-α [TNF-α], interleukin [IL]-1β, IL-6, IL-8 and IL-10) in lipopolysaccharide (LPS)-induced THP-1 cells and to compare the effects in human-derived monocytes vs macrophages.

2.2 Methods and materials

2.2.1 Chemicals and reagents

Pg-3-glc was purchased from Extrasynthese (Genay, France). PCA (3,4-dihydroxybenzoic acid), 4-HBA, PGA (2,4,6-trihydroxybenzaldehyde), LPS from Escherichia coli, phorbol 12-myristate 13-acetate (PMA), methanol and formic acid were purchased from Sigma-Aldrich (Dorset, United Kingdom). RPMI 1640 culture medium with 300 mg/L glutamine, foetal calf serum (FCS) and antibiotics (penicillin and streptomycin) were purchased from Lonza (Basel, Switzerland). The cytometric bead array kit to analyze cytokine concentrations was purchased from BD Biosciences (Oxford, United Kingdom). Pg-3-glc, PCA, 4-HBA and PGA were dissolved in acidified methanol (2% formic acid) to a concentration of 10 mM and stored at −70 °C and away from light. RPMI culture medium was stored at 4 °C, FCS and antibiotics were defrosted upon delivery, aliquoted and stored at −20 °C until use.

2.2.2 Preparation and culture of THP-1 cells

THP-1 cells (human monocytic leukemia, ECACC 88081201) were cultured in RPMI 1640 culture medium supplemented with 100 UI/mL streptomycin, 100 µg/mL penicillin and 10% (v/v) FCS at 37 °C in a humidified atmosphere of 5 % CO_2 and kept at a density of 2-9 x 10^5 cells/mL. For the experiments, cells were seeded in 24 well plates at a density of 1 x 10^6 cells/mL. For differentiation into macrophages, cells were exposed to PMA at a final concentration of 0.1 µM for 72 h [42], following which the medium was removed and replaced with fresh medium containing the desired concentration of polyphenols. Polyphenols were added to provide final concentrations of 0.08, 0.31, 1.25, 5 and 20 µM. Polyphenols were added in 20 µL of 10.98% methanol, 0.22% formic acid to produce a final concentration of 0.22%

methanol and 0.004% formic acid in culture. The final culture volume was 1 mL. Control cultures contained 0.22% methanol and 0.004% formic acid but no polyphenols. LPS (20 µL; 1 µg/mL final concentration) was added to stimulate cytokine production.

Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. At the end of the incubation, plates were centrifuged at 260 x g for 5 min and culture supernatants were collected and stored in aliquots at −20 °C until analysis.

2.2.3 Measurement of cytokine concentrations

Concentrations of TNF-α, IL-1β, IL-6, IL-8 and IL-10 in the culture supernatants were measured using a cytometric bead array kit from BD Biosciences (Oxford, United Kingdom) according to the manufacturer's instructions. Data were acquired on a BD FACS Canto™ II flow cytometer and analyzed using the BD FCAP Array v3 software. Limits of detection of the cytokine assays were 0.13 pg/mL (IL-10), 1.2 pg/mL (TNF-α and IL-8), 1.6 pg/mL (IL-6) and 2.3 pg/mL (IL-1β).

2.2.4 Cytotoxicity

To determine whether test compounds had any cytotoxic effects, cell viability of THP-1 monocytes as well as THP-1 macrophages was assessed using Trypan blue staining.

2.2.5 Statistical analyses

Results are expressed as percentage of cytokine production versus control (no polyphenols) and shown as means with their standard deviations (SD). One-way ANOVA was performed to determine whether test compounds affected the cytokine production, followed by Dunnett as post hoc analysis versus control group. Statistical analysis was performed using SPSS 21 (IBM

Corporation, New York, USA) and a lowered $P<0.01$ was considered significant to account for multiple comparisons.

2.3 Results

2.3.1 Effects of Pg-3-glc, PCA, 4-HBA and PGA on viability of THP-1 cells

There were no cytotoxic effects of the studied compounds on either THP-1 monocytes or macrophages at any of the tested doses (data not shown).

2.3.2 Effects of Pg-3-glc, PCA, 4-HBA and PGA on cytokine production by THP-1 monocytes

Stimulation with LPS increased IL-1β production 135-fold, TNF-α production 105-fold, IL-6 production 470-fold, IL-8 production 640-fold and IL-10 production 5-fold. PCA significantly reduced IL-6 production at 0.31, 1.25 and 20 μM compared to the control cultures (all $P<0.01$, Table 1). PGA also significantly inhibited IL-6 production and while the effects were only significant at 5 and 20 μM ($P<0.01$ and $P<0.001$ respectively, Table 1), they were slightly more potent (production was lowered by 25-35% compared to 20% for PCA) and appeared to be concentration-dependent. There was no significant effect of any tested compound, at concentrations up to 20 μM, on the production of IL-1β, TNF-α, IL-8 or IL-10 by THP-1 monocytes (Table 1).

2.3.3 Effects of Pg-3-glc, PCA, 4-HBA and PGA on cytokine production by THP-1 macrophages

Stimulation with LPS increased IL-1β production 10-fold, TNF-α production 45-fold, IL-6 production 220-fold, IL-8 production 80-fold and IL-10 production 5-fold. PGA was the only compound that induced significant changes in cytokine production. PGA at the highest dose tested (20 μM) significantly lowered IL-6 production ($P<0.001$, Table 2), similar to the monocyte results and with a similar magnitude of effect. Furthermore, it also significantly decreased IL-10 production (20 μM; $P<0.01$; Table 2), an effect not observed in THP-1 monocytes. There was no significant effect of any of the tested compounds, at concentrations up to 20 μM, on the production of IL-1β, TNF-α or IL-8 by THP-1 macrophages (Table 2).

Table 1 Effect of Pg-3-glc, PCA, 4-HBA and PGA on IL-1β, TNF-α, IL-6, IL-8 and IL-10 production by THP-1 monocytes

	Cytokine production (% of control)				
	IL-1β	**TNF-α**	**IL-6**	**IL-8**	**IL-10**
Pg-3-glc					
0 μM	100	100	100	100	100
0.08 μM	91.8 ± 18.9	114.2 ± 20.5	85.6 ± 17.5	108.6 ± 15.6	113.4 ± 12.4
0.31 μM	92.7 ± 10.7	110.8 ± 20.3	78.2 ± 13.2	102.3 ± 19.6	104.0 ± 8.9
1.25 μM	93.2 ± 8.4	112.9 ± 20.1	78.4 ± 11.8	96.1 ± 13.0	102.9 ± 10.4
5.00 μM	92.3 ± 9.6	106.4 ± 19.4	84.5 ± 10.4	101.9 ± 12.1	102.5 ± 8.0
20.00 μM	96.4 ± 13.9	101.1 ± 20.0	88.5 ± 18.6	103.9 ± 19.4	98.0 ± 4.9
PCA					
0 μM	100	100	100	100	100
0.08 μM	94.5 ± 7.8	103.5 ± 18.9	87.3 ± 7.0	110.3 ± 13.0	107.0 ± 6.9
0.31 μM	97.7 ± 7.5	93.4 ± 15.3	77.8 ± 9.7 **	98.2 ± 7.1	104.6 ± 5.9
1.25 μM	104.9 ± 5.2	92.9 ± 10.7	77.2 ± 10.2 **	106.0 ± 9.0	105.4 ± 7.0
5.00 μM	95.2 ± 12.8	85.7 ± 7.9	88.3 ± 15.9	100.5 ± 14.9	99.0 ± 9.5
20.00 μM	89.3 ± 19.3	85.9 ± 11.0	79.8 ± 14.1 **	102.4 ± 10.1	93.6 ± 7.6
4-HBA					
0 μM	100	100	100	100	100
0.08 μM	83.4 ± 7.6	104.2 ± 21.2	99.1 ± 14.5	94.0 ± 18.2	99.9 ± 8.6
0.31 μM	97.7 ± 10.4	109.3 ± 22.5	90.9 ± 5.1	91.3 ± 15.7	104.8 ± 8.9
1.25 μM	97.3 ± 15.3	110.4 ± 26.4	91.9 ± 3.6	90.9 ± 16.5	101.8 ± 6.9
5.00 μM	100.2 ± 8.6	107.2 ± 19.5	85.1 ± 21.6	93.5 ± 17.8	103.7 ± 8.0
20.00 μM	105.7 ± 13.0	114.2 ± 16.5	93.4 ± 25.0	90.2 ± 22.2	102.3 ± 7.1
PGA					
0 μM	100	100	100	100	100
0.08 μM	97.2 ± 11.6	111.4 ± 21.3	90.0 ± 16.2	103.2 ± 13.7	110.6 ± 13.1
0.31 μM	102.1 ± 7.4	107.5 ± 21.4	91.9 ± 17.2	96.8 ± 21.1	104.6 ± 11.0
1.25 μM	106.5 ± 11.8	112.9 ± 18.9	79.7 ± 10.6	97.0 ± 13.8	101.2 ± 9.0
5.00 μM	110.4 ± 12.3	117.0 ± 22.1	73.6 ± 6.9 **	95.2 ± 20.6	97.1 ± 9.2
20.00 μM	106.2 ± 12.6	111.6 ± 21.7	65.9 ± 17.6 ***	94.6 ± 19.3	95.6 ± 6.1

THP-1 monocytes (1 x 10^6 cells/mL) were treated with Pg-3-glc, PCA, 4-HBA or PGA at concentrations of 0-20 μM, prior to lipopolysaccharide stimulation (1 μg/mL) and incubated for 24 h at 37 °C. Cytokine concentrations in the culture supernatants were measured using a cytometric bead array kit. Results are expressed as percentage of cytokine concentration versus control (no polyphenols). Data are represented as the mean ± SD of six independent experiments. Data were analyzed by one-way ANOVA and Dunnett post hoc analysis, where applicable, and a lowered $P<0.01$ was considered significant to account for multiple comparisons. Statistically significant differences are denoted as $^{**}P<0.01$ vs control; $^{***}P<0.001$ vs control. 4-HBA, 4-hydroxybenzoic acid; IL, interleukin; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; TNF-α, tumor necrosis factor-α.

Table 2 Effect of Pg-3-glc, PCA, 4-HBA and PGA on IL-1β, TNF-α, IL-6, IL-8 and IL-10 production by THP-1 macrophages

	Cytokine production (% of control)				
	IL-1β	**TNF-α**	**IL-6**	**IL-8**	**IL-10**
Pg-3-glc					
0 μM	100	100	100	100	100
0.08 μM	113.2 ± 18.8	117.3 ± 31.0	113.5 ± 24.7	111.4 ± 12.8	100.9 ± 17.4
0.31 μM	111.6 ± 15.1	115.8 ± 22.2	108.0 ± 21.6	106.5 ± 12.2	87.3 ± 15.1
1.25 μM	108.9 ± 12.3	112.4 ± 16.0	101.4 ± 24.1	105.8 ± 16.9	88.0 ± 16.8
5.00 μM	107.8 ± 16.7	110.5 ± 24.5	101.6 ± 20.1	103.4 ± 16.1	80.1 ± 13.5
20.00 μM	99.2 ± 14.7	106.0 ± 18.1	107.7 ± 19.8	102.9 ± 11.8	84.3 ± 16.0
PCA					
0 μM	100	100	100	100	100
0.08 μM	102.7 ± 9.3	117.2 ± 23.2	116.2 ± 25.2	106.5 ± 16.1	100.8 ± 12.0
0.31 μM	103.8 ± 10.0	101.0 ± 18.6	111.7 ± 22.9	104.4 ± 14.1	85.0 ± 24.9
1.25 μM	100.8 ± 6.8	96.5 ± 26.0	101.8 ± 23.6	101.7 ± 12.5	84.1 ± 22.2
5.00 μM	100.9 ± 10.8	101.7 ± 17.5	107.2 ± 23.9	98.7 ± 12.0	83.2 ± 19.6
20.00 μM	99.1 ± 9.9	100.6 ± 22.9	88.6 ± 19.4	103.7 ± 12.0	77.8 ± 27.2
4-HBA					
0 μM	100	100	100	100	100
0.08 μM	108.7 ± 17.9	115.3 ± 28.7	107.9 ± 21.4	111.3 ± 13.6	106.2 ± 13.5
0.31 μM	107.4 ± 17.1	112.2 ± 27.7	104.2 ± 17.0	103.4 ± 16.6	93.1 ± 15.0
1.25 μM	110.2 ± 18.2	109.6 ± 29.3	101.1 ± 14.4	102.6 ± 19.5	90.8 ± 19.0
5.00 μM	109.9 ± 16.8	97.8 ± 27.3	97.4 ± 27.4	103.0 ± 15.2	88.5 ± 16.5
20.00 μM	112.2 ± 18.4	109.7 ± 27.9	94.7 ± 25.8	95.2 ± 16.1	94.8 ± 11.6
PGA					
0 μM	100	100	100	100	100
0.08 μM	106.6 ± 7.4	113.9 ± 21.7	109.5 ± 17.6	110.9 ± 16.4	110.3 ± 21.2
0.31 μM	111.4 ± 11.9	107.6 ± 18.0	95.7 ± 18.6	104.8 ± 11.9	77.6 ± 15.3
1.25 μM	112.3 ± 8.7	100.5 ± 18.7	79.7 ± 16.0	103.1 ± 21.2	72.1 ± 16.0
5.00 μM	110.9 ± 16.3	98.5 ± 21.4	71.8 ± 19.1	106.3 ± 12.3	70.9 ± 19.1
20.00 μM	113.7 ± 17.9	110.5 ± 23.7	58.8 ± 25.6 ***	115.6 ± 23.2	65.4 ± 26.2 **

THP-1 monocytes (1 x 10^6 cells/mL) were converted to macrophages through exposure to 0.1 μM phorbol-12-myristate-13-acetate for 72 h. THP-1 macrophages were treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at concentrations of 0-20 μM, prior to lipopolysaccharide stimulation (1 μg/mL) and incubated for 24 h at 37 °C. Cytokine concentrations in the culture supernatants were measured using a cytometric bead array kit. Results are expressed as percentage of cytokine concentration versus control (no polyphenols). Data are represented as the mean ± SD of six independent experiments. Data were analyzed by one-way ANOVA and Dunnett post hoc analysis, where applicable, and a lowered $P<0.01$ was considered significant to account for multiple comparisons. Statistically significant differences are denoted as $^{**}P<0.01$ vs control; $^{***}P<0.001$ vs control. 4-HBA, 4-hydroxybenzoic acid; IL, interleukin; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; TNF-α, tumor necrosis factor-α.

2.4 Discussion

Inflammation is present in all stages of atherosclerosis [16, 43-48], with monocytes and macrophages playing a critical role as they secrete a number of pro-inflammatory cytokines contributing to the development of atherosclerosis and plaque destabilization [16].

This screening study aimed to compare the effects of the parent anthocyanin, Pg-3-glc, and the physiologically relevant plasma metabolites, PCA, 4-HBA and PGA, on cytokine secretion by LPS-stimulated THP-1 cells. There were modest anti-inflammatory effects of some of the tested compounds in THP-1 monocytes, with PCA and PGA inhibiting IL-6 production, but there were no effects on TNF-α, IL-1β, IL-8 and IL-10 and there were no effects of the other compounds. The effects in macrophages were slightly different. Whilst PGA inhibited IL-6 production by THP-1 derived macrophages, it also inhibited IL-10 production, which was not observed in the THP-1 monocytes.

IL-6 is generally classified as a pro-inflammatory cytokine as it induces the liver to produce acute phase proteins and stimulates myloid cell differentiation and smooth muscle cell proliferation [16]. The inhibition of LPS-stimulated IL-6 secretion by PCA and PGA in the monocyte cultures and PGA in the macrophage cultures is a novel observation. Importantly, the inhibitory effect of PCA was observed at the physiologically attainable [35, 36, 38] low micromolar concentrations. Taken together with other data in the literature, the current results contribute to the suggestion that PCA acts on multiple cell types involved in IL-6 secretion during atherosclerosis. PCA was previously demonstrated to inhibit IL-6 secretion by endothelial cells (HUVEC) stimulated with oxidized LDL or CD40 ligand [13] and by LPS-stimulated human monocyte-derived dendritic cells [10], although the latter experiment applied supraphysiological concentrations of PCA. Whilst the bioactivity of PGA on IL-6 was a novel observation, the effect was only significant at the higher 5 and 20 μM concentrations. As levels

in circulation remain below micromolar concentration (even after anthocyanin consumption), this finding is likely of limited physiological relevance [37, 38].

IL-10 is generally classified as an anti-inflammatory cytokine as it impedes Th1 responses, stimulates regulatory T cell proliferation and differentiation, suppresses macrophage activation and inhibits several pro-inflammatory cytokines, chemokines and growth factors [16]. PGA decreased the production of IL-10 in THP-1 macrophages, suggesting a pro-inflammatory tendency. This finding was unexpected and seems to contrast the IL-6-reducing (i.e. anti-inflammatory) effect of PGA. However, it is important to note that the IL-10 decreasing effect was observed only at high concentrations that are unlikely to be achieved *in vivo*. To our knowledge, no other studies have investigated the effect of PGA on IL-10 production by monocytes or macrophages. There is evidence to suggest that the inhibition of IL-6 and IL-10 production could be linked as it has been reported that IL-6 enhances IL-10 levels, so inhibition of IL-6 production could indirectly inhibit production of IL-10 [49]. There were no effects of any of the other tested compounds on IL-10 levels, which is in line with results from other groups testing the effect of PCA in human monocyte-derived dendritic cells [10] and Pg-3-glc, PCA and 4-HBA in THP-1 monocytes [12]. Beneficial effects on IL-10 production were observed in human leukocytes with tea-derived polyphenols [50], suggesting differential bioactivity of different polyphenol classes, although effects were only observed at the higher, supraphysiological concentrations of tea-derived polyphenols (10 and 20 μM).

It is important to note in this context that the consumption of other dietary sources might also contribute to the circulatory pool of PCA as it is not a pelargonidin-specific metabolite. The presence of PCA in plasma has been reported following ingestion of other anthocyanins [38, 51, 52] and the flavonol quercetin [53]. It is also naturally present in several other dietary sources, such as chicory, olives, raspberries, dates and onions [2, 54, 55].

4-HBA had no effect on the secretion of any of the cytokines tested and there are only two comparable studies, which report mixed findings. Consistent with data from the current investigation, 4-HBA at 0.1-10 μM was previously reported to have no effect on TNF-α secretion by LPS-stimulated THP-1 monocytes. Interestingly, however, the same publication reports an inhibitory effect on TNF-α when 4-HBA was coincubated with PCA or PCA plus vanillic acid, none of which was bioactive in isolation. Therefore, this study is indicative of additive/synergistic effects between polyphenols, highlighting the importance of studying physiologically relevant combinations in further studies [12]. Furthermore, this study observed a reduction in IL-1β levels following incubation with 4-HBA. This is in contrast to the present study, despite both studies employing similar concentrations of 4-HBA, the same cell line (THP-1 monocytes) and similar culture conditions (24 h stimulation with 1 μg/mL LPS). The only apparent difference was that no preincubation with test compounds was conducted in the current experiment. In peripheral blood mononuclear cells, 1 μM 4-HBA had no effect on IL-1β or IL-6 levels in accordance with the present data, but a modest 10% increase in TNF-α levels was observed, which could be related to the different cell type used and different conditions of culture. The discrepant findings regarding the effect of 4-HBA on IL-1β and TNF-α merit further investigation.

In this study only some of the tested compounds modulated cytokine secretion, namely PCA and PGA, whose effects were targeted towards IL-6 and IL-10, while having no impact on the other cytokines. In contrast, 4-HBA and Pg-3-glc did not affect any of the cytokines tested. These results suggest that at least two hydroxyl groups (either *ortho* or *para* to each other) might be required for bioactivity. In line with this suggestion, potent radical scavenging activity of polyphenols was previously linked to the presence of an *ortho*-dihydroxy group [56] and was furthermore correlated with the number of hydroxyl groups on the B-ring [57]. In order to

identify chemical structures / properties required for anti-inflammatory effects, screening studies with a larger number of related compounds are clearly required.

The molecular mechanism of action for the inhibitory action of strawberry polyphenols and their metabolites on cytokine production was not explored in the present study. However, there is evidence to suggest that the mechanism might include inhibition of transcription factors nuclear factor-kappa B (NF-κB) [9-11, 17, 22, 25, 26], activator protein 1 [58] and/or reduction of mitogen-activated protein kinase activity [11, 25]. It is important to note, however, that NF-κB pathway modulation has recently been questioned in a study where a number of phenolic compounds were reported to selectively modulate TNF-α levels while having no effect on other NF-κB-controlled cytokines (IL-1β and IL-10) [12] and the present investigation lends credence to this hypothesis.

2.5 Conclusion

In conclusion, the data suggest that PCA may possess anti-inflammatory properties through modulation of IL-6 production. Importantly, this action was observed at physiologically attainable concentrations, although the effect was modest and limited to monocytes. The IL-6 and IL-10-reducing effects of PGA were only observed at the higher, supraphysiological concentration and are thus considered of limited physiological relevance. Pg-3-glc and 4-HBA had no effect on any of the tested cytokines. Future screening studies should focus on screening a larger number of related compounds in order to identify chemical structures or properties required for anti-inflammatory effects and underlying mechanisms.

2.6 Acknowledgments

The authors declare no conflicts of interest related to this experiment.

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Chapter 3 Effects of a strawberry-derived anthocyanin and its metabolites on phagocytosis and lipopolysaccharide-stimulated cytokine production by human whole blood cultures

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The authors' contributions were as follows: AMA, JPES, and PY designed the experiment; AMA and K M conducted t he experiment; AMA performed the l aboratory a nd statistical analyses; AMA wrote the manuscript; all authors read and approved the final manuscript

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Abstract

This screening study investigated the effects of the predominant strawberry anthocyanin, pelargonidin-3-O-glucoside (Pg-3-glc), and three of its plasma metabolites (4-hydroxybenzoic acid, protocatechuic acid and phloroglucinaldehyde [PGA]) on phagocytosis, oxidative burst and the production of selected pro- and anti-inflammatory cytokines in a whole blood culture model. For the assessment of phagocytosis and oxidative burst activity of monocytes and neutrophils, whole blood was pre-incubated in the presence or absence of the test compounds at concentrations up to 5 μM, followed by analysis of phagocytic and oxidative burst activity using commercially available test kits. For the cytokine analysis, diluted whole blood was stimulated with lipopolysaccharide in the presence or absence of the test compounds at concentrations up to 5 μM. Concentrations of selected cytokines (tumor necrosis factor-α [TNF-α], interleukin [IL]-1β, IL-6, IL-8 and IL-10) were determined using a cytometric bead array kit. There were no effects of any of the test compounds on phagocytosis of opsonized or non-opsonized *E. coli* or on oxidative burst activity. Pg-3-glc and PGA at 0.08 μM increased the concentration of IL-10 ($P<0.01$ and $P<0.001$, respectively), but there was no effect on TNF-α, IL-1β, IL-6 and IL-8 and there were no effects of the other compounds. In conclusion, this screening study demonstrated a lack of effect of these compounds on the opsonization, engulfment and subsequent destruction of bacteria. Pg-3-glc and PGA, at physiologically relevant concentrations, had anti-inflammatory properties; however, effects were modest, only observed at the lowest dose tested and limited to IL-10.

Keywords: Anthocyanin, Cytokine, Immune function, Inflammation, Pelargonidin-3-O-glucoside, Phagocytosis, Strawberry

3.1 Introduction

Anthocyanins belong to the flavonoid group of polyphenols and are abundant in berry fruits. In recent years, anthocyanins have attracted considerable interest as dietary constituents that may provide a variety of health benefits to humans, including cardiovascular disease prevention, obesity control, alleviation of diabetes, improvement of vision and memory and increased immune defenses [1, 2]. An anthocyanin-rich elderberry extract was demonstrated to exert antimicrobial and antiviral activity in *vitro* towards human pathogenic respiratory bacteria and influenza viruses, although mechanisms were unclear [3]. Leukocytes play a crucial role in host defense against bacterial and viral infections [4] and several leukocyte functions have been reported to be influenced by flavonoids, although some of the data is conflicting. Blueberries [5], an anthocyanin-rich juice [6] and purple sweet potato leaves [7] were reported to increase numbers and activity of natural killer cells, but there was no effect of red wine, dealcoholized red wine or red grape juice [8, 9]. Similarly, lymphocyte proliferation and interleukin (IL)-2 secretion by activated lymphocyte was increased upon consumption of an anthocyanin-rich juice [6] and purple sweet potato leaves [7], but not affected by red wine, dealcoholized red wine or red grape juice [8, 9]. Red wine, dealcoholized red wine or red grape juice also had no effect on phagocytosis by neutrophils and monocytes [8, 9]. In an animal model, red wine anthocyanins increased phagocytic activity at 25 and 50 mg/kg body weight, but decreased phagocytic activity at doses ≥200 mg/kg body weight [10]. There is a lack of evidence from human studies on other anthocyanin-rich interventions. In mice, pyogallol-type green tea polyphenols increased phagocytic activity *in vitro* [11] and a polyphenol-rich cereal fraction increased phagocytic activity and increased production of reactive oxygen species and superoxide anion [12], but a number of anthocyanin- and flavonoid-rich fruits were reported to diminish reactive oxygen species production [13]. Thus, there is limited and conflicting data regarding the influence of flavonoids on the phagocytic process.

Cytokines are also a critical component of immune defense, but, on the other hand, inappropriate or excessive production of pro-inflammatory cytokines has been linked with the pathogenesis of a number of chronic inflammatory diseases [4]. Several cell culture studies have explored the effect of anthocyanins to modulate cytokine production and other parameters of immune function, but the majority were conducted using the unmetabolized parent anthocyanins, often at high doses [3, 10, 13-26], which may not be physiologically relevant. Strawberries constitute a popular fruit and they are particularly rich in anthocyanins, predominantly pelargonidin-3-O-glucoside (Pg-3-glc) [27]. Glucuronidated pelargonidin has been reported as the predominant metabolite in three pharmacokinetic studies [28-30], but there is ambiguity regarding the position of glucuronidation and glucuronidated pelargonidin compounds are currently commercially unavailable and hence cannot be tested in cell culture models. 4-hydroxybenzoic acid (4-HBA) and protocatechuic acid (PCA) have also been reported in plasma following strawberry consumption in low micromolar concentration (0.1-2 μM) [30-32]. In addition, it is likely that phloroglucinaldehyde (PGA) might appear in plasma following strawberry consumption, as it is a A-ring degradant, reported in plasma upon anthocyanin consumption in low to high nanomolar concentration (20-600 nM) [33, 34]. There is very little data on the effects of these physiologically relevant compounds. Furthermore, most *in vitro* work has been conducted using cell lines, but whole blood cultures more closely represent physiological conditions [35, 36]. This screening study therefore characterized the effect of the parent anthocyanin Pg-3-glc and three physiologically relevant plasma metabolites on phagocytosis, oxidative burst and the production of selected pro- and anti-inflammatory cytokines (tumor necrosis factor-α [TNF-α], IL-1β, IL-6, IL-8 and IL-10) in a whole blood culture model.

3.2 Methods and materials

3.2.1 Subjects

Ten healthy volunteers (8 females and 2 males) aged 42 to 63 years were recruited for this screening study. Volunteers were selected according to the following inclusion criteria: 40–65 years old, good general health, absence of diabetes, cancer, liver cirrhosis, asplenia, other acquired or congenial immunodeficiency, HIV, any kind of inflammatory, auto-immune disease or connective tissue disease. The exclusion criteria were use of anti-inflammatory or immunomodulating medication, use of antibiotics within 3 months, vaccination within 3 months, participation in another drug or nutritional research study within 3 months and alcoholism or drug misuse. Subjects were asked to follow a low-flavonoid diet for 24 h prior to the blood sample collection. On the day of the study volunteers came fasted to the Hugh Sinclair Unit of Human Nutrition of the University of Reading. Fasted blood was collected into sodium heparin vacutainer tubes (Greiner Bio-One Ltd, Gloucestershire, UK). Written informed consent was obtained from all subjects. The work was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the University of Reading Research Ethics Committee (Project reference 10/05).

3.2.2 Materials

Pg-3-glc was purchased from Extrasynthese (Genay, France). PCA (3,4-dihydroxybenzoic acid), 4-HBA, PGA (2,4,6-trihydroxybenzaldehyde), lipopolysaccharides from *E. coli* (LPS), methanol and formic acid were purchased from Sigma-Aldrich (Dorset, United Kingdom). RPMI 1640 culture medium, fetal calf serum (FCS) and antibiotics (penicillin and streptomycin) were purchased from Lonza (Basel, Switzerland). The cytometric bead array kit to analyze

cytokine concentrations was purchased from BD Biosciences (Oxford, United Kingdom). Phagoburst™ and Phagotest™ kits as well as the nonopsonized *E. coli* bacteria used with the Phagotest™ kit were manufactured by Glycotope Biotechnology (Heidelberg, Germany). Pg-3-glc, PCA, 4-HBA and PGA were dissolved in acidified methanol (2% formic acid) to a concentration of 10 mM and stored at −70 °C and away from light. Further dilutions of the test compounds were prepared freshly on each study day by dilution in RPMI medium with added FCS and antibiotics. RPMI culture medium was stored at 4 °C, FCS and antibiotics were defrosted upon delivery, aliquoted and stored at −20 °C until use.

3.2.3 Whole blood culture for phagocytosis and oxidative burst capacity

Heparinized whole blood samples were pre-incubated with the test compounds (Pg-3-glc, PCA, 4-HBA, PGA or RPMI 1640 medium as control) at 4 different concentrations (0.08, 0.31, 1.25 and 5 µM) at 37 °C for 4 h in 15 x 75 mm tubes.

3.2.4 Whole blood culture for cytokine analysis

Heparinized whole blood was diluted 6:10 with RPMI 1640 medium supplemented with FCS and antibiotics. The diluted blood (1 mL/well) was placed into 24-well tissue culture plates. Working solutions of the test compounds (prepared with supplemented RPMI 1640 medium) were added to provide final concentrations of 0.08, 0.31, 1.25 and 5 µM. Respective volumes of RPMI 1640 medium were added to control cultures (no polyphenols). LPS (1 µg/mL final concentration) was added to stimulate cytokine production. Cultures were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. At the end of the culture period, plates were centrifuged at 260 x g for 5 min. Culture supernatants were collected and stored in aliquots at −20 °C until analysis.

3.2.5 Measurement of leukocyte phagocytosis and oxidative burst capacity

Phagocytic and oxidative burst activity of monocytes and neutrophils were analyzed using commercially available test kits (Phagotest™ and Phagoburst™) following the instructions of the manufacturer. The percentage of neutrophils or monocytes engaged in phagocytosis of *E. coli* bacteria (opsonized and nonopsonized) and oxidative burst activity and the mean fluorescence intensity (MFI) were acquired on a BD FACS Canto™ II flow cytometer. Data was analyzed using DIVA software.

3.2.6 Measurement of cytokine concentrations

Concentrations of TNF-α, IL-1β, IL-6, IL-8 and IL-10 in the culture supernatants were measured using a cytometric bead array kit from BD Biosciences (Oxford, United Kingdom) according to the manufacturers' instructions. The intensity of the fluorescence signal was acquired on a BD FACS Canto™ II flow cytometer and data analyzed using the BD FCAP Array v3 software. Limits of detection of the cytokine assays are 0.13 pg/mL (IL-10), 1.2 pg/mL (TNF-α and IL-8), 1.6 pg/mL (IL-6) and 2.3 pg/mL (IL-1β).

3.2.7 Statistical analyses

Results are expressed as percentage of phagocytic activity/oxidative burst activity/cytokine production versus control (no polyphenols) and shown as means with their standard deviations (SD). One-way ANOVA was performed to determine whether each test compound affected the phagocytic activity/oxidative burst activity/cytokine production, followed by Dunnett as post hoc analysis versus control group where appropriate. Statistical analysis was performed using

SPSS 21 (IBM Corporation, New York, USA) and a lowered $P<0.01$ was considered significant to account for multiple comparisons.

3.3 Results

3.3.1 Effects of Pg-3-glc, PCA, 4-HBA and PGA on phagocytic and oxidative burst activity

None of the test compounds significantly affected the overall percentage of neutrophils or monocytes engaged in phagocytosis of opsonized (Table 1) or non-opsonized (Table 2) *E. coli* bacteria. A high degree of inter-subject variability was notable. Further, there were no significant effects of any of the test compounds on the MFI, which indicates degree of phagocytic activity (Table 1 and Table 2).

Similarly, none of the test compounds significantly changed the overall percentage of neutrophils or monocytes engaged in oxidative burst (Table 3) and there was no significant effect on the MFI as a measure of degree of oxidative burst activity (Table 3).

3.3.2 Effects of Pg-3-glc, PCA, 4-HBA and PGA on cytokine production

Stimulation with LPS increased IL-1β production 11-fold, TNF-α production 9-fold, IL-6 production 2-fold, IL-8 production 1-fold and IL-10 production 5-fold. Pg-3-glc and PGA at the lowest dose tested (0.08 μM) significantly increased IL-10 production compared to the control cultures ($P<0.01$ and $P<0.001$ respectively, Table 4). There was no significant effect of any tested compound, at concentrations up to 5 μM, on the production of IL-1β, TNF-α, IL-6 or IL-8 by human whole blood cultures (Table 4).

Table 1 Effect of Pg-3-glc, PCA, 4-HBA and PGA on phagocytic activity of nonopsonized *E. coli* bacteria in human whole blood cultures

	Neutrophils		Monocytes	
	% Phagocytizing	MFI	% Phagocytizing	MFI
Pg-3-glc				
0 μM	100	100	100	100
0.08 μM	115.8 ± 16.3	106.4 ± 21.1	105.0 ± 30.7	93.6 ± 11.4
0.31 μM	119.1 ± 42.1	108.8 ± 29.0	103.3 ± 41.0	94.0 ± 11.3
1.25 μM	111.9 ± 21.5	104.9 ± 22.2	98.6 ± 19.7	99.2 ± 12.4
5.00 μM	120.5 ± 32.4	107.4 ± 20.2	119.7 ± 42.0	99.5 ± 9.8
PCA				
0 μM	100	100	100	100
0.08 μM	122.2 ± 41.5	109.4 ± 23.7	111.6 ± 34.3	96.4 ± 5.9
0.31 μM	124.6 ± 41.1	108.1 ± 27.8	103.1 ± 32.5	94.0 ± 8.1
1.25 μM	115.6 ± 17.2	106.7 ± 11.8	106.6 ± 29.5	95.9 ± 12.9
5.00 μM	123.2 ± 33.1	110.4 ± 20.3	107.7 ± 21.7	93.5 ± 8.4
4-HBA				
0 μM	100	100	100	100
0.08 μM	129.6 ± 36.9	112.5 ± 22.5	116.2 ± 25.5	96.2 ± 10.5
0.31 μM	125.1 ± 41.5	111.5 ± 28.5	109.3 ± 20.1	95.3 ± 7.5
1.25 μM	124.8 ± 43.6	113.0 ± 27.7	108.1 ± 31.7	93.6 ± 11.2
5.00 μM	125.0 ± 38.2	110.4 ± 26.5	110.6 ± 31.8	91.9 ± 6.7
PGA				
0 μM	100	100	100	100
0.08 μM	114.5 ± 24.2	99.3 ± 18.4	106.0 ± 25.6	94.1 ± 7.1
0.31 μM	113.7 ± 21.3	96.5 ± 15.6	103.8 ± 19.7	88.3 ± 12.3
1.25 μM	102.3 ± 23.6	95.1 ± 12.4	94.9 ± 39.3	90.6 ± 14.7
5.00 μM	112.8 ± 28.3	100.7 ± 17.1	100.5 ± 31.8	88.9 ± 10.4

Human whole blood (n = 10) was treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at concentrations of 0-5 μM, for 4 h at 37 °C. Phagocytic activity was analyzed using the Phagotest™ test kit. Results are expressed as percentage of phagocytic activity versus control (no polyphenols). Data are represented as the mean ± SD. Data were analyzed by one-way ANOVA and a lowered $P<0.01$ was considered significant to account for multiple comparisons. There were no statistically significant changes. 4-HBA, 4-hydroxybenzoic acid; MFI, mean fluorescence intensity; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde.

Table 2 Effect of Pg-3-glc, PCA, 4-HBA and PGA on phagocytic activity of opsonized *E. coli* bacteria in human whole blood cultures

	Neutrophils		Monocytes	
	% Phagocytizing	MFI	% Phagocytizing	MFI
Pg-3-glc				
0 µM	100	100	100	100
0.08 µM	105.5 ± 47.0	96.7 ± 22.7	99.1 ± 24.1	97.9 ± 14.1
0.31 µM	119.0 ± 47.0	99.2 ± 18.2	98.5 ± 33.3	92.0 ± 9.1
1.25 µM	112.9 ± 33.4	102.5 ± 16.7	95.3 ± 21.0	93.3 ± 13.6
5.00 µM	104.5 ± 13.9	97.8 ± 14.7	101.5 ± 19.7	97.7 ± 12.1
PCA				
0 µM	100	100	100	100
0.08 µM	111.3 ± 13.8	100.2 ± 18.6	101.6 ± 13.2	91.1 ± 11.8
0.31 µM	105.1 ± 23.1	98.9 ± 19.0	101.2 ± 17.9	94.6 ± 16.5
1.25 µM	99.2 ± 11.7	92.1 ± 10.3	100.8 ± 12.6	93.8 ± 11.3
5.00 µM	101.5 ± 15.4	97.6 ± 17.1	98.8 ± 19.2	95.2 ± 13.5
4-HBA				
0 µM	100	100	100	100
0.08 µM	106.7 ± 17.1	97.3 ± 14.4	96.8 ± 20.2	90.4 ± 10.1
0.31 µM	107.5 ± 18.4	91.3 ± 18.5	100.1 ± 16.2	88.9 ± 11.5
1.25 µM	102.3 ± 17.9	91.3 ± 10.8	96.0 ± 17.1	86.2 ± 13.1
5.00 µM	102.0 ± 18.9	96.1 ± 18.8	99.8 ± 20.7	89.5 ± 16.1
PGA				
0 µM	100	100	100	100
0.08 µM	104.2 ± 51.4	97.8 ± 20.9	95.8 ± 27.7	93.6 ± 16.2
0.31 µM	121.9 ± 48.5	104.9 ± 19.3	105.6 ± 39.0	97.5 ± 16.2
1.25 µM	116.3 ± 31.2	100.9 ± 17.8	97.8 ± 10.4	93.1 ± 9.2
5.00 µM	128.3 ± 53.6	101.8 ± 20.4	101.6 ± 18.2	93.1 ± 9.1

Human whole blood (n = 10) was treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at concentrations of 0-5 µM, for 4 h at 37 °C. Phagocytic activity was analyzed using the Phagotest™ test kit. Results are expressed as percentage of phagocytic activity versus control (no polyphenols). Data are represented as the mean ± SD. Data were analyzed by one-way ANOVA and a lowered $P<0.01$ was considered significant to account for multiple comparisons. There were no statistically significant changes. 4-HBA, 4-hydroxybenzoic acid; MFI, mean fluorescence intensity; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde.

Table 3 Effect of Pg-3-glc, PCA, 4-HBA and PGA on oxidative burst activity in human whole blood cultures

	Neutrophils		Monocytes	
	% Oxidizing	MFI	% Oxidizing	MFI
Pg-3-glc				
0 µM	100	100	100	100
0.08 µM	93.9 ± 14.9	108.3 ± 40.0	105.2 ± 19.9	100.5 ± 28.0
0.31 µM	102.7 ± 11.9	118.9 ± 45.1	107.6 ± 20.5	98.5 ± 23.4
1.25 µM	93.5 ± 11.2	97.6 ± 35.5	92.2 ± 18.4	91.0 ± 21.9
5.00 µM	93.9 ± 16.4	108.0 ± 42.1	102.9 ± 23.7	100.8 ± 27.9
PCA				
0 µM	100	100	100	100
0.08 µM	95.7 ± 19.8	94.4 ± 24.2	98.3 ± 36.8	109.7 ± 34.8
0.31 µM	87.5 ± 19.9	90.5 ± 13.4	95.3 ± 33.3	97.4 ± 23.8
1.25 µM	95.7 ± 12.3	95.6 ± 19.2	99.7 ± 14.5	93.6 ± 26.4
5.00 µM	92.4 ± 14.5	86.1 ± 27.6	90.4 ± 18.8	83.9 ± 27.4
4-HBA				
0 µM	100	100	100	100
0.08 µM	100.7 ± 15.8	102.2 ± 39.1	95.1 ± 18.1	103.2 ± 39.5
0.31 µM	101.5 ± 16.0	99.4 ± 32.6	99.0 ± 22.8	101.9 ± 35.4
1.25 µM	102.7 ± 15.5	95.1 ± 28.6	97.8 ± 20.5	87.5 ± 22.4
5.00 µM	90.1 ± 22.0	82.4 ± 25.0	89.6 ± 29.7	92.9 ± 20.1
PGA				
0 µM	100	100	100	100
0.08 µM	99.9 ± 12.2	105.1 ± 36.0	96.3 ± 23.5	98.8 ± 26.9
0.31 µM	103.4 ± 13.8	101.6 ± 29.7	96.8 ± 21.3	94.9 ± 26.7
1.25 µM	98.4 ± 18.8	101.2 ± 27.1	100.4 ± 14.5	103.4 ± 51.1
5.00 µM	96.6 ± 13.9	109.4 ± 45.7	104.4 ± 20.0	96.6 ± 24.6

Human whole blood (n = 10) was treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at concentrations of 0-5 µM, for 4 h at 37 °C. Oxidative burst activity was analyzed using the Phagoburst™ test kit. Results are expressed as percentage of oxidative burst activity versus control (no polyphenols). Data are represented as the mean ± SD. Data were analyzed by one-way ANOVA and a lowered $P<0.01$ was considered significant to account for multiple comparisons. There were no statistically significant changes. 4-HBA, 4-hydroxybenzoic acid; MFI, mean fluorescence intensity; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde.

Table 4 Effect of Pg-3-glc, PCA, 4-HBA and PGA on IL-1β, TNF-α, IL-6, IL-8 and IL-10 production in human whole blood cultures

	Cytokine production (% of control)				
	IL-1β	**TNF-α**	**IL-6**	**IL-8**	**IL-10**
Pg-3-glc					
0 μM	100	100	100	100	100
0.08 μM	95.8 ± 11.2	100.2 ± 26.1	98.5 ± 5.4	106.2 ± 14.5	118.0 ± 22.6**
0.31 μM	96.6 ± 11.1	96.9 ± 14.6	93.9 ± 6.7	93.2 ± 10.4	98.5 ± 6.3
1.25 μM	93.0 ± 10.4	96.1 ± 12.0	94.7 ± 9.1	89.8 ± 10.7	98.2 ± 7.6
5.00 μM	90.3 ± 13.8	96.6 ± 12.0	93.5 ± 9.2	88.9 ± 11.0	100.1 ± 6.4
PCA					
0 μM	100	100	100	100	100
0.08 μM	99.0 ± 7.6	108.0 ± 13.5	102.0 ± 10.6	106.0 ± 23.6	107.1 ±14.4
0.31 μM	99.4 ± 7.1	95.0 ± 16.1	94.8 ± 9.8	89.5 ± 15.0	97.4 ± 7.0
1.25 μM	99.8 ± 10.7	95.3 ± 20.3	94.6 ± 10.5	89.8 ± 14.7	101.5 ± 5.9
5.00 μM	96.8 ± 9.8	97.7 ± 16.7	95.3 ± 7.0	90.3 ± 13.5	102.7 ± 10.5
4-HBA					
0 μM	100	100	100	100	100
0.08 μM	91.2 ± 7.9	101.0 ± 8.8	96.0 ± 7.0	108.5 ± 21.0	103.4 ± 11.1
0.31 μM	92.9 ± 7.6	99.6 ± 8.8	94.4 ± 7.2	98.1 ± 15.6	97.0 ± 7.6
1.25 μM	90.8 ± 7.6	98.4 ± 9.2	94.7 ± 9.2	95.9 ± 14.9	95.0 ± 8.1
5.00 μM	92.4 ± 17.0	103.8 ± 22.2	98.7 ± 8.7	95.3 ± 12.5	97.2 ± 10.9
PGA					
0 μM	100	100	100	100	100
0.08 μM	93.4 ± 14.6	95.6 ± 26.8	100.6 ± 11.6	109.3 ± 13.8	123.8 ± 25.6***
0.31 μM	94.4 ± 8.7	102.0 ± 19.1	96.0 ± 9.7	96.4 ± 10.2	104.0 ± 7.8
1.25 μM	98.4 ± 11.2	100.2 ± 14.9	97.3 ± 9.4	94.9 ± 11.9	97.1 ± 6.9
5.00 μM	108.4 ± 13.7	98.4 ± 13.3	93.2 ± 8.6	103.8 ± 15.4	98.6 ± 10.8

Human whole blood (n = 10) was treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at concentrations of 0 -5 μM, prior to lip opolysaccharide s timulation (1 μg/mL) and incubated for 24 h a t 3 7 °C. C ytokine concentrations in the culture supernatants were measured using a cytometric bead array kit. Results are expressed as percentage of cytokine concentration versus control (no polyphenols). Data are represented as the mean ± SD. Data were analyzed by one-way ANOVA and Dunnett post hoc analysis, where applicable, and a lowered $P<0.01$ was considered significant to account for multiple comparisons. Statistically significant differences are denoted as **$P<0.01$ v s c ontrol; ***$P<0.001$ v s c ontrol. 4 -HBA, 4 -hydroxybenzoic a cid; IL, in terleukin; P CA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; TNF-α, tumor necrosis factor-α.

3.4 Discussion

The current screening study investigated, for the first time, the effects of the strawberry-derived anthocyanin Pg-3-glc and three of its physiologically relevant plasma metabolites on phagocytosis, oxidative burst and the production of selected pro- and anti-inflammatory cytokines in a screening approach using whole blood cultures. There were no effects of any of the test compounds on phagocytosis of opsonized or non-opsonized *E. coli* or on oxidative burst activity, indicating a lack of effect of these compounds on the opsonization, engulfment or subsequent destruction of bacteria. In agreement with this data, some human intervention studies report no significant effects of anthocyanin-rich red wine, red grape juice [8, 9] or quercetin [37-40] on phagocytic ability of monocytes and neutrophils. However, higher doses of red wine anthocyanins did increase phagocytic activity in mice [10], suggesting that the outcomes of animal and human studies may differ and that dose might be important.

Once pathogens are engulfed by phagocytes (phagocytosis), they are destroyed in part by the production of reactive oxygen metabolites in a process termed oxidative burst [4]. In the current screening study, there were no effects of any of the test compounds on oxidative burst activity upon *E. coli* stimulation. In contrast, a reduction in hydrogen peroxide production was reported by a raspberry fruit extract in phorbol-12-myristate-13-acetate (PMA)-stimulated J774 murine macrophages, but the effects were only observed at higher extract concentrations and were less pronounced in arachidonic acid-stimulated macrophages [41]. Similarly, diminished production of reactive oxygen species production was reported by a number of anthocyanin- and flavonoid-rich fruits by opsonized zymosan-activated phagocytes, but no effects were observed when PMA was used as stimuli [13]. This study used higher polyphenol doses compared to the current experiment, where doses may have been too low to have an effect. As there was no effect of PMA, the authors concluded a modulation of the signaling cascade upstream to activation of protein kinase C, which is targeted by PMA. The two studies have

also used a different technique for ROS measurement. Another important consideration that may contribute to discrepant findings between studies might be the nature of the stimulus used. Emerging data suggests that the phagocytic immune response is governed by the type of stimulus [42]. It is important to note that interpretation of oxidative burst data is not straightforward. On the one hand, oxidative burst is involved in the destruction of pathogens upon phagocytosis and thus represents a critical component of immune defense [4], but it can also be harmful to tissues and contribute to the pathogenesis of chronic health conditions [13], especially if there are insufficient antioxidant defenses.

Cytokines are a critical component of immune defense, but, on the other hand, inappropriate or excessive production of TNF-α, IL-1β, IL-6 and IL-8 has been linked with the pathogenesis of a number of chronic inflammatory diseases [4]. IL-10, on the other hand, is a predominantly anti-inflammatory cytokine and would be expected to be associated with reduced atherosclerosis by suppressing macrophage activation and inhibiting several pro-inflammatory cytokines, chemokines and growth factors [43]. In the current experiment, the increased IL-10 production by Pg-3-glc and PGA could be interpreted as a modest anti-inflammatory effect. Interestingly, in the current experiment the IL-10 increasing effect of Pg-3-glc and PGA was only observed at the lowest dose tested (0.08 μM). Although the validity and interpretation of this effect remains to be confirmed, it could also indicate the presence of an inverted U-shaped response curve as has previously been reported for polyphenols. For example, trends for U-shaped or inverted U-shaped associations were previously observed in a cell model between vanillic acid and heme oxygenase-1 protein expression [44], in a human intervention study between blueberry beverage consumption and flow-mediated dilation [45] and in an epidemiological study between tea consumption and coronary heart disease mortality [46]. The same four compounds tested in the current experiment, Pg-3-glc, PCA, 4-HBA or PGA, had no significant effects on IL-10 production by THP-1 monocytes (Chapter 2), whilst in THP-1

macrophages, PGA at 20 µM decreased the production of IL-10 (Chapter 2), but this dose is unphysiologically high and may already reflect the other end of the inverted U-shaped response curve. To our knowledge, no other studies have investigated the effect of PGA on IL-10 production by monocytes or macrophages. There were no effects of any of the other test compounds on IL-10 levels, which is in line with results on the effect of PCA in human monocyte-derived dendritic cells [25] and PCA and 4-HBA in THP-1 monocytes [47]. In the latter study, Pg-3-glc did not alter IL-10 production, which is in contrast to the current data. However that study only tested a dose of 1 µM and the lack of effect was consistent with the observation that Pg-3-glc at 0.31-5.00 µM had no effect in the current study.

There is evidence from *in vitro* studies to suggest that particular structural characteristics might be required for phagocytosis-enhancing effects. A cell line study (using 1,25-dihydroxyvitamin D3-differentiated HL60 cells) concerned with the effect of green tea polyphenols suggested that a pyrogallol-type B-ring and/or a galloyl group are required to increase phagocytic activity [11]. This could provide an explanation for the results of the current experiment as these structural characteristics were absent from the tested compounds. However, this observation was only based on a screen of six compounds and therefore requires confirmation, ideally in screening studies with a larger number of related compounds to clearly identify chemical structures or properties required for phagocytosis-enhancing effects.

The common cold is the world's most prevalent acute illness, with adults experiencing approximately two to four colds per year [48]. A reduction of common cold symptoms has been demonstrated after intervention with Juice Plus, a dietary supplement composed of juice powder concentrate from fruits and vegetables [49]. Indeed it has been suggested that flavonoids might improve immune defenses. However, the current data do not support the suggestion that changes in opsonization, phagocytosis or oxidative burst of pathogens are the underlying mechanism of action, at least not in the case of Pg-3-glc and its metabolites.

Alternative mechanisms of action could include, for example, improved natural killer cell activity, lymphocyte proliferation and cytokine production by lymphocytes. There is some suggestion that these mechanisms might be targeted by flavonoids, although the evidence is not conclusive and requires further research.

3.5 Conclusion

In conclusion, there was no effect of the strawberry-derived anthocyanin Pg-3-glc or three of its physiologically relevant plasma metabolites on phagocytosis or oxidative burst activity in an *in vitro* human whole blood culture model. The data suggest that PGA and Pg-3-glc at physiologically attainable concentrations may possess anti-inflammatory properties; however, effects were modest, only observed at the lowest dose tested (0.08 μM) and limited to IL-10. None of the test compounds had any effect on IL-1β, TNF-α, IL-6 and IL-8. Subsequent studies should explore other immunomodulatory effects of dietary anthocyanins.

3.6 Acknowledgments

The authors declare no conflicts of interest related to this experiment.

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Chapter 4 Acute effects of an anthocyanin-rich blackcurrant beverage on markers of cardiovascular disease risk in healthy adults: a randomized, double-blind, placebo-controlled, crossover trial

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The authors' contributions were as follows: AMA, JPES, and PY designed the study; AMA, KA, DK, ISI, and RZ conducted the human study; GS and GC performed the polyphenol and metabolite analysis of plasma and urine samples, TB-M developed the methodology for the microparticle measurements, VS-K provided advice on the nitric oxide analyzer, HA performed the polyphenol analysis of the study beverages under supervision of PK, AMA analyzed data and performed statistical analyses; AMA and PY wrote the manuscript; all authors read and approved the final manuscript.

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Abstract

Background: Epidemiological evidence suggests an inverse association between anthocyanin consumption and cardiovascular disease risk. Modulation of vascular function and hemostasis may contribute to this, but there is limited clinical evidence.

Objective: The present study investigated the acute effects of an anthocyanin-rich blackcurrant beverage in response to a high-fat meal on selected markers of cardiovascular disease risk in healthy middle-aged men and women.

Design: Twenty-three volunteers aged 39.9 ± 8.1 years with a BMI of 22.9 ± 2.3 kg/m^2 completed this double-blind, randomized, placebo-controlled, crossover trial. Volunteers consumed either 200 mL blackcurrant beverage (744 mg polyphenols, 711 mg anthocyanins, 32 mg procyanidins) or a placebo, together with a high-fat breakfast, and the postprandial vascular response was compared. The primary endpoints were the assessment of vascular function by flow-mediated dilation (FMD) and the inhibition of collagen- and adenosine diphosphate-induced platelet aggregation. Blood pressure, digital volume pulse waveforms, circulating numbers of endothelium- and platelet-derived microparticles, plasma concentration of nitrite, nitrate and interleukin (IL)-8 were also evaluated.

Results: The anthocyanin-rich blackcurrant beverage significantly improved FMD (difference of 1.1% over the course of the 6 h post-treatment period, $P_{treatment}<0.000001$, $P_{treatment*time\ interaction}=0.425$), agonist-induced platelet aggregation, IL-8 and systolic blood pressure although the integral area under the curve was not significantly different for the latter. There were no significant effects of the beverage on diastolic blood pressure, arterial stiffness, plasma concentrations of nitrite, nitrate, or numbers of endothelial or platelet microparticles.

Conclusion: The present study reports for the first time that an anthocyanin-rich blackcurrant beverage can beneficially ameliorate changes in vascular function and markers of CVD risk in response to a high-fat meal relative to the placebo. Improvements were observed in FMD, platelet function, systolic blood pressure and IL-8 concentrations, which could contribute to CVD prevention.

This trial was registered at clinicaltrials.gov as NCT02459756.

Keywords: Anthocyanins, Blackcurrant, Blood pressure, Inflammation, Microparticles, Platelet aggregation, Vascular function

4.1 Introduction

Anthocyanins are abundant in berry fruits and belong to the flavonoid group of polyphenols. A cardioprotective benefit of flavonoid-rich foods has been demonstrated in several studies (1). However, the most convincing clinical evidence exists only for a few flavonoid-rich products (chocolate, black and green tea), which have been extensively studied. Consumption of these flavonoid sources has been reported to exert beneficial effects on some risk factors for cardiovascular disease (CVD), such as LDL cholesterol, blood pressure (BP), and also on vascular function and platelet aggregation. Epidemiological evidence has also linked consumption of anthocyanins with a lower risk of CVD (2-4) and CVD risk markers (5-7), but only a few clinical intervention trials have investigated the effect of anthocyanin-rich interventions on CVD risk markers (8). Blueberry polyphenols have been demonstrated to improve vascular function (flow-mediated dilation, FMD) in healthy men, and the biphasic increase in FMD observed at 1-2 and 6 h post-treatment was paralleled by the appearance of phenolic compounds in plasma and inhibition of neutrophil NADPH oxidase activity (9). However, the blueberry intervention delivered other polyphenol classes (predominantly procyanidins and chlorogenic acid) in addition to anthocyanins and the nature of their bioactivity is thus unclear.

There are several further potential mechanisms by which anthocyanins may reduce the risk of CVD, including modulation of (i) platelet activation and aggregation, (ii) microparticle release, (iii) cytokine production and (iv) arterial stiffness. There is some evidence on the predictive value of platelet activity on CVD risk (10-12) and a recent review of human randomized controlled trials (RCTs) concludes that there is strong evidence for an anti-platelet effect of flavan-3-ol-rich cocoa (products) and grape seed extract, particularly after acute consumption (13), but little is known about the effect of anthocyanin-rich interventions.

Elevated numbers of circulating microparticles, which are small plasma membrane vesicles shed from the surface of a variety of stimulated and/or apoptotic cells, are an emerging risk marker for CVD (14), suggested to indicate vascular injury and inflammation (15). Consumption of cocoa polyphenols reduces the number of circulating endothelial-derived microparticles (EMPs) in patients with coronary artery disease (16) as well as obese and overweight women, although no effects were observed in normal weight women (17). To our knowledge, no RCTs have investigated the effect of anthocyanins on the number of circulating microparticles. There is also little information regarding the effects of anthocyanins following a vascular challenge, such as a high-fat meal. As such, the aim of the present study was to investigate the postprandial effects of blackcurrant-derived anthocyanins on a range of CVD risk markers in a healthy, middle-aged population.

4.2 Subjects and Methods

4.2.1 Subjects

Volunteers were recruited from Reading and surrounding areas (England) by email, posters and internet advertisements; the participant flow diagram is depicted in Figure 1. A total of twenty-three volunteers completed the study (39.87 ± 8.13 years old; 11 females). Their baseline characteristics are summarized in Table 1. Eligible for participation were healthy, non-smoking men and women aged 30-55 years, with a normal to overweight BMI (20-30 kg/m^2). Exclusion criteria included: systolic blood pressure (SBP) >140 mm Hg; diastolic blood pressure (DBP) >90 mm Hg; total cholesterol >6.2 mmol/L; anemia (hemoglobin concentrations <130 g/L in men and <120 g/L in women); presence of diabetes mellitus, heart problems, stroke, vascular, inflammatory, kidney, liver, pancreas or gastrointestinal diseases; usage of medication for hyperlipidemia, hypertension, hypercoagulation or inflammatory conditions;

usage of aspirin >2 x/wk; unable to abstain from aspirin ingestion for 14 days prior to each study visit; usage of antibiotics in the previous 3 months before the study; participation in intense aerobic exercise (>3 x 20 min/wk); pregnancy or breastfeeding; usage of phytochemical, antioxidant or fish oil supplements, veganism, alcohol misuse or intakes >21 units/wk for men and >15 units/wk for women or participation in another clinical trial. Full blood count parameters and markers of liver and kidney function were confirmed to be within the normal range prior to entry into the study.

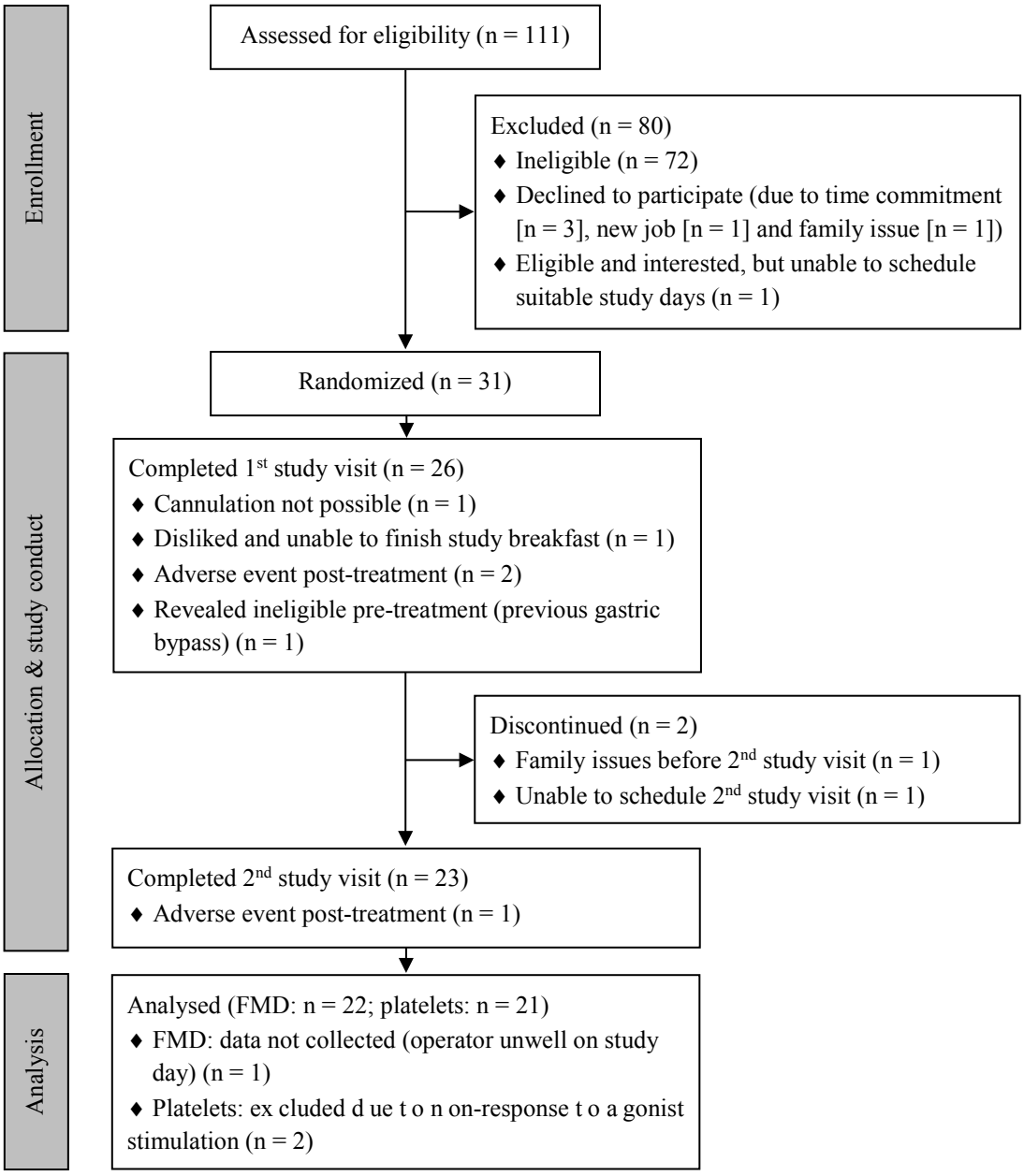

Figure 1 Participant flow. FMD, flow-mediated dilation.

The study was conducted at the Hugh Sinclair Unit of Human Nutrition at the University of Reading, from June to November 2015. Written informed consent was obtained from all subjects. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, approved by the University of Reading Research Ethics Committee (reference: UREC 14/17) as well as GlaxoSmithKline and registered at clinicaltrials.gov as NCT02459756.

Table 1 Clinical characteristics of subjects who completed the study (n = 23; 12 males)

Baseline characteristic	Mean ± SD
Age (years)	39.9 ± 8.1
BMI (kg/m^2)	22.9 ± 2.3
Body fat (%)	23.0 ± 5.9
Systolic blood pressure (mm Hg)	118.2 ± 11.9
Diastolic blood pressure (mm Hg)	71.5 ± 8.1
Serum total cholesterol (mmol/L)	4.9 ± 1.2
Serum fasting glucose (mmol/L)	4.9 ± 1.0

4.2.2 Study design

A randomized, double-blind, placebo-controlled crossover intervention trial was performed in which volunteers were asked to consume each of the following 2 treatments in random order: blackcurrant beverage (200 mL containing 711 mg anthocyanins, Table 2) and a matched placebo beverage. Treatment order randomization was conducted by minimization using the stratification variables gender, age and BMI. The study visits took place at the Hugh Sinclair

Unit of Human Nutrition at the University of Reading separated by four weeks for women (to control for possible effects of the menstrual hormones on vascular function) or at least three weeks for men. Subjects were asked to refrain from exercise, caffeine and alcohol and to follow a low-flavonoid diet for 24 h before the visit and throughout the study day. Subjects were provided with a low-fat, low-flavonoid meal for consumption on the evening before each study day. Subjects arrived at the clinical unit after an overnight fast (12 h) and a 24 h dietary recall was conducted to verify compliance to the low-flavonoid diet. A cannula was inserted into the forearm and blood samples were collected at baseline and at 1, 2, 4, 6 and 24 h post-treatment. Polyphenol & metabolite analysis was performed at all time points; IL-8 and nitrite/nitrate analysis at baseline and at 1, 2, 4 and 6 h post-treatment; platelet aggregation and microparticle analysis at baseline and at 2 and 4 h post-treatment. Vascular measurements (FMD, BP and DVP [digital volume pulse]) were performed at baseline and at 2, 4 and 6 h post-treatment. FMD and BP were additionally measured at 1 h post-treatment. Urine was collected at baseline and at 0-1, 1-2, 2-4, 4-6 and 6-24 h post-treatment for polyphenol & metabolite analysis. The total volume of urine produced during each time period was recorded. After the baseline measurements were taken, subjects consumed, within 15 min, the test beverage together with a standardized, high-fat, low-flavonoid breakfast (756.1 kcals, 52.3 g fat, 57.9 g carbohydrates and 11.3 g protein) consisting of two croissants with butter. A standardized low-flavonoid lunch (665.5 kcals, 30.0 g fat, 81.1 g carbohydrates and 15.1 g protein) consisting of a soft cheese sandwich, ready salted crisps and two shortbread biscuits was provided after the 2 h measurements and a low-fat, low-flavonoid meal for dinner. Low-nitrate water (Buxton) was provided to subjects during the whole intervention period as required. Researchers involved in the measurement and assessment of study outcomes were blinded to the allocation of treatment orders.

Table 2 Composition of the test beverages (per serving)

Parameter	Blackcurrant beverage	Placebo beverage
Polyphenols (mg)		
Total polyphenols	744	0
Total anthocyanins	711	0
Delphinidin-3-O-rutinoside	349	0
Cyanidin-3-O-rutinoside	207	0
Delphinidin-3-O-glucoside	102	0
Cyanidin-3-O-glucoside	33	0
Petunidin-3-O-rutinoside	7.7	0
Petunidin 3-(6-coumaroyl)-glucoside	7.7	0
Peonidin-3-O-rutinoside	4,9	0
Total procyanidins	32	0
Monomers	6.9	0
Oligomers	26	0
Macronutrients (g)		
Sucrose	1.13	1.13
Glucose	0.62	0.62
Fructose	0.72	0.72
Maltodextrin	0.46	0.46
Protein	0.15	0
Fat	0.01	0
Organic acids		
Total (g)	0.50	0.50 [1]
Vitamin C (mg)	1.72	1.72
Minerals (mg)		
Potassium	34.73	0
Calcium	12.51	0
Magnesium	4.31	0
Sodium	1.03	0
Phosphorus	5.22	0
Other		
Artificial berry flavoring (g)	0.20	0.20
Low-nitrate water (Buxton) (mL)	200	200
Fiber (g)	0.07	0

Quantification of polyphenols was conducted by HPLC at the University of Norwich, while information on other constituents in the blackcurrant powder was provided by BerryPharma. [1] Except for vitamin C there was no detailed information available on the type and proportion of organic acids in the blackcurrant powder, hence available data on the organic acid content in blackcurrants from the literature were used (18) to formulate the placebo beverage (0.3% vitamin C, 6% malic acid and 94% citric acid).

4.2.3 Test beverages

Table 2 details the composition of the test beverages. The intervention beverage contained 744 mg polyphenols, of which 711 mg were anthocyanins (49% delphinidin-3-O-rutinoside, 29% cyanidin-3-O-rutinoside, 14% delphinidin-3-O-glucoside, 5% cyanidin-3-O-glucoside and 3% other anthocyanins) and 32 mg procyanidins and was comprised of spray-dried blackcurrant powder (*Ribes nigrum*, BerryPharma, Leichligen, Germany, product 70140015, batch L 14IV04342, stored in the dark at −20 °C until use), sucrose (Tate & Lyle) and artificial blackcurrant flavoring (International Flavours & Fragrances, Haverhill, UK) dissolved in 200 mL of low-nitrate water. The placebo beverage was devoid of polyphenols and was matched for sugars (sucrose, glucose and fructose [all Myprotein]) and organic acids (citric acid [Sigma Aldrich], vitamin C and malic acid [both Myprotein]). In addition, artificial blackcurrant flavoring was added to both beverages to mask taste differences. Study beverages were analyzed for anthocyanin and procyanidin content, the main polyphenol classes that have previously been reported in blackcurrants (19). Quantification of anthocyanins and procyanidins was conducted by HPLC at the University of Norwich, while information on other constituents in the blackcurrant powder was provided by BerryPharma. The powdered beverage ingredients were weighed out and packaged as individual servings at the University of Reading, coded A or B by an independent researcher and stored in the dark at −20 °C until use. Encoding was broken after all data analysis had been completed. On study days, the beverages were prepared by an independent researcher immediately before consumption by dissolving in 200 mL of low-nitrate water. Beverages were served in identical non-transparent lidded containers with a black straw to conceal color differences.

4.2.4 Blood and urine collection

Blood samples were collected into K_3EDTA tubes (for microparticles and polyphenols and their metabolites), lithium heparin tubes (for IL-8 and nitrate/nitrite) and sodium citrate tubes (for platelet aggregation). After blood sample collection, sodium citrate tubes were stored at room temperature, whereas the other tubes were kept on ice. Blood samples for the analysis of IL-8 and polyphenols and metabolites were centrifuged at 1700 x g for 15 min at 4 °C. Blood samples for the analysis of nitrate/nitrite were centrifuged at 2040 x g for 3 min at 4 °C and centrifugation was started at exactly 5 min after blood collection, due to the reported nitrite instability in drawn whole blood (20). Samples were divided into aliquots and stored at −20 °C (for IL-8 and nitrate/nitrite analysis) or −70 °C (for polyphenol and metabolite analysis). Plasma for polyphenol and metabolite analysis was acidified with 2% formic acid (Sigma Aldrich) before storage to prevent anthocyanin degradation (9). Urine was supplemented with 100 mg ascorbic acid (Sigma Aldrich) per 500 mL urine and acidified with formic acid to pH 2.4 before storage (21).

4.2.5 Assessment of vascular function and BP

Measurements were performed in a quiet, temperature-controlled (24 ± 2 °C) room. The subject lay quietly for several minutes before the measurements were performed.

Brachial artery FMD was measured according to established guidelines (22). A Phillips CX50 integrated ultrasound system (Phillips, Amsterdam, Netherlands) utilizing a 15-7 MHz transducer (L15-7io Broadband Compact Linear Arrray transducer, Phillips, Amsterdam, Netherlands) was used in combination with a semi-automated analysis system (Brachial Analyzer, Medical Imaging Applications, Iowa City, USA). Briefly, the brachial artery was imaged at 2–10 cm proximal to the antecubital fossa in the longitudinal plane. After recording

baseline arterial diameter for 60 s, reactive hyperemia was induced by 5 min of lower arm occlusion through inflation of a cuff to 220 mm Hg. Data collection continued for 5 min after cuff release. A single researcher analyzed all image files. The peak diameter was defined as the mean of the three biggest diameters obtained after the occlusion was released. FMD was calculated as the percentage change in the brachial artery diameter from baseline to the peak diameter after cuff release. Each frame was analyzed in triplicate and the average used in the statistical analysis. All FMD measurements were performed by the same trained operator.

DVP waveforms were measured in the semi-supine position using a PulseTrace PCA 2 device (CareFusion, San Diego, CA, USA) by finger photoplethysmography. The device automatically analyzes DVP waveforms and calculates the stiffness index (DVP-SI) and the reflection index (DVP-RI). DVP-SI is a measure of large artery stiffness (23), while DVP-RI is a measure of the vascular tone of the small arteries (24).

BP was measured on the upper arm using an automated sphygmomanometer (Omron M6 Comfort, Omron Corporation, Kyoto, Japan) with the subject in a supine position after 5 min of rest. Three consecutive measurements were recorded and the average used in the statistical analysis.

4.2.6 Assessment of agonist-induced *ex vivo* platelet aggregation

Citrated blood for platelet function measurement was collected via a cannula at baseline as well as 2 and 4 h post-treatment and measurements performed within 90 min of blood collection. Platelet-rich plasma (PRP) was prepared by centrifugation at 102 x g for 20 min. Following the removal of PRP, another centrifugation step was performed at 1413 x g for 10 min to provide platelet-poor plasma (PPP). Platelets in PRP were counted using a Z2 Coulter Counter (Beckman Coulter, Brea, USA). Platelet aggregation was induced in PRP (225 uL) with two agonists: collagen (0.5 and 1 µg/mL final concentration; Takeda, Linz, Austria) and ADP (10

and 100 µM final concentration; Sigma Aldrich). Measurements were performed in a Chronolog optical platelet aggregometer (Chrono-log, Havertown, USA) with constant stirring at 1200 rpm and at 37 °C.

4.2.7 Assessment of microparticles

Numbers of circulating EMPs and platelet-derived microparticles (PMPs) were quantified as previously described (25). In brief, PPP was stained with CD42b conjugated to fluorescein isothiocyanate and CD31 conjugated to phycoerythrin antibodies in TruCount tubes (all from BD Biosciences, Oxford, UK). After an incubation period, samples were diluted with phosphate-buffered saline and data acquired on a BD Accuri flow cytometer (BD Biosciences, Oxford, UK) using the gating strategy described in detail in Wu, et al. (25). EMPs were defined as CD31+CD42b− and PMPs as CD31+CD42b+. Values are expressed as counts per microliter of PPP.

4.2.8 Assessment of nitrite and nitrate

Plasma was deproteinized by the addition of methanol (Sigma Aldrich) and nitrite and nitrate concentrations measured using a HPLC-based nitric oxide analyzer (ENO-30, Eicom, San Diego, USA). In brief, nitrite and nitrate are separated using a reverse-phase column (NO-PAK, Eicom), followed by a reduction of nitrate to nitrite in a reduction column (NO-RED, Eicom). Nitrite then reacts with the Griess reagent forming a colored diazo compound that is measured spectrophotometrically based on its absorbance at 540 nm. The detection limit for nitrite and nitrate were 0.05-0.075 µM and 3.125 µM respectively. While all nitrate results were above detection limit, 31% of nitrite data were below detection limit. The intra-assay CV for nitrite and nitrate were 18.4 and 3.3%, respectively.

4.2.9 Assessment of IL-1β, TNF-α, IL-6, IL-8 and IL-10 concentration

Plasma concentrations of IL-8 were measured using an enhanced sensitivity cytometric bead array kit from BD Biosciences (Oxford, UK) according to the manufacturers' instructions. Data were acquired on a BD FACS Canto II flow cytometer (BD Biosciences, Oxford, UK) and analyzed using the BD FCAP Array v3 software. Limits of detection of these cytokine assays are 0.274 pg/mL. Analysis of IL-1β, TNF-α, IL-6 and IL-10 was discontinued following sub-analysis of 5 volunteers where most data were below the limit of detection.

4.2.10 Power calculation

The sample size calculation was performed according to Julious, et al. (26) for the primary endpoints of brachial artery FMD and agonist-induced platelet aggregation. For the former, 15 subjects would provide 80% power in detecting a difference of 0.7% absolute difference in brachial artery FMD with α=0.05 and a within-subject standard deviation of 0.6. For platelet function, 21 subjects would provide 80% power in detecting a difference of 6% or greater in agonist-induced platelet aggregation with α=0.05 and a within-subject standard deviation of 5.5%.

4.2.11 Statistical analyses

Differences from baseline in study outcomes were analyzed using a linear mixed model for a 2-period crossover study with 2, 3 or 4 repeated measures post-treatment. The model included volunteer as random factors and treatment, time and sequence as fixed effects. Incremental areas under the curve (iAUC) were calculated using the trapezoid rule and paired t tests carried out to compare treatment differences. Model assumptions were assessed through diagnostic plots of residuals. Differences in baseline values were compared with a paired t test. Results

are expressed as means ± SEMs. A P value <0.05 was considered significant and statistical analyses were performed using SPSS 21 (IBM Corporation, New York, USA).

4.3 Results

Twenty-three subjects completed both study visits (Figure 1). There were three adverse events (vomiting post-treatment during the study day and in one case this was coupled with an uncomfortable cannula). These subjects were withdrawn from the study, but the symptoms were observed with both beverages. No serious adverse events were reported.

Baseline characteristics of subjects who completed the study are presented in Table 1. According to inclusion/exclusion criteria, volunteers were healthy, normal- or overweight and without additional CVD risk factors. There were no significant differences in baseline measures of FMD, platelet aggregation, nitrite, nitrate, SBP, DBP, DVP-SI, DVP-RI, PMPs or EMPs between placebo and intervention days (Table 3), but baseline IL-8 concentration was significantly higher on days where the blackcurrant beverage was consumed (paired t test, 2230 pg/mL vs 1913 pg/mL; P=0.008).

4.3.1 FMD

There were highly significant effects of time and treatment (both P<0.000001), but no significant treatment x time interaction (P=0.425; Figure 2). FMD was significantly higher over the course of the study day after consumption of the blackcurrant beverage compared with placebo (ANOVA: P<0.000001, t test [iAUC]: P<0.0001). The magnitude of difference in FMD between both beverages over the course of the study day was 1.1%. Although there was no significant treatment x time interaction (P=0.425) and the FMD response following consumption of the two beverages was different across all time points (apart from baseline),

Figure 2 is suggestive of a greater difference at 2 h. For both beverages, FMD decreased following consumption of the high-fat breakfast and the test beverage, with the maximum decrease being observed at 1 h post-treatment, and although there was no significant treatment x time interaction, Figure 2 suggests that FMD decreased to a lesser extent after the blackcurrant beverage than after the placebo beverage (decrease of 1.61 ± 0.33% versus 0.75 ± 0.33%). This was followed by an FMD peak at 2 h (blackcurrant beverage) and 4 h (placebo), where FMD values returned to baseline for placebo, but remained around 1% higher than baseline for the blackcurrant beverage (Figure 2).

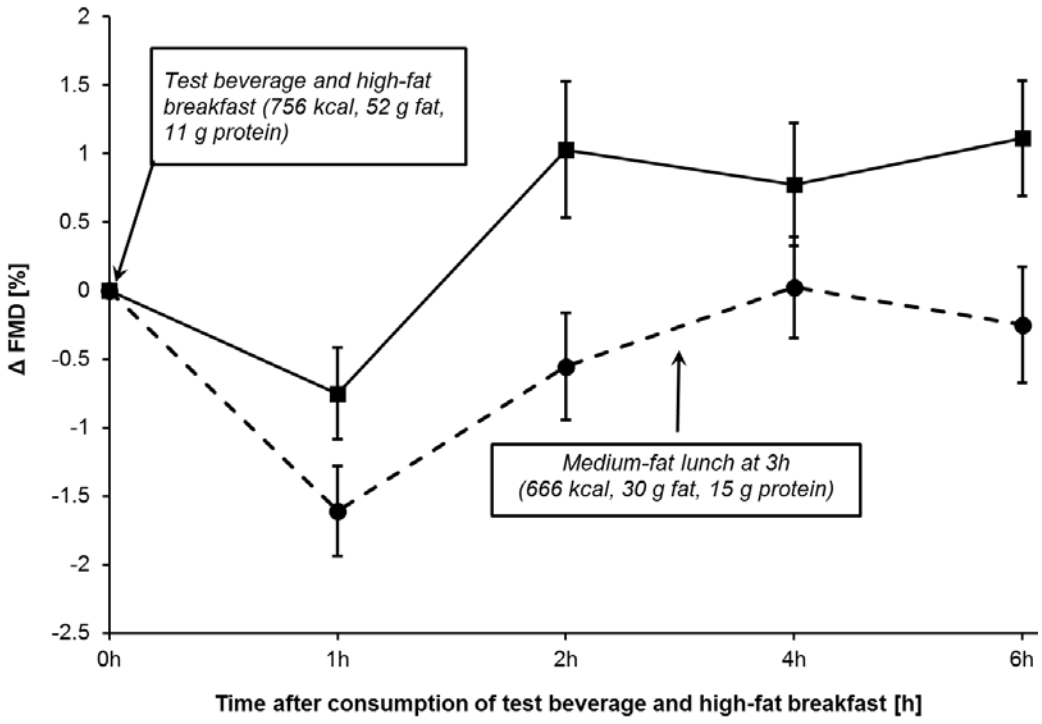

Figure 2 Change from baseline in FMD (mean ± SEM) after consumption of the blackcurrant beverage containing 711 mg anthocyanins (solid line) or placebo (dashed line) together with a high-fat breakfast (50 g fat) (n = 22). *Volunteers consumed a medium-fat lunch 3 h after they had consumed the breakfast and test beverage. Differences from baseline in FMD values were analyzed using a linear mixed model with treatment (2 treatments: blackcurrant compared to placebo beverage) and time (4 levels) as factors: the main effects for treatment and time were significant (both P<0.000001), but there was no significant treatment x time interaction (P=0.425). FMD, flow-mediated dilation.*

4.3.2 DVP

The blackcurrant beverage had no effect on DVP-SI or DVP-RI (Table 3).

4.3.3 BP

There were significant effects of time and treatment for SBP (P=0.004 and P=0.015, respectively), but no significant treatment x time interaction (P=0.807; Table 3). The linear mixed model indicated that SBP was significantly higher over the course of the study day after consumption of the placebo beverage compared with the intervention ($P_{treatment}$=0.015), but the iAUCs were not significantly different (P=0.152). Regarding DBP, there was no significant effect of time (P=0.290) and no significant treatment x time interaction (P=0.997), although there was a trend for higher DBP after placebo consumption ($P_{treatment}$=0.054; Table 3), but the iAUCs were not significantly different (P=0.315).

4.3.4 Agonist-induced platelet aggregation

There was a significant effect of treatment on platelet aggregation stimulated by both ADP (10 and 100 µM) and 1 µg/mL collagen (P=0.00003, 0.0001 and 0.0005, respectively, Table 4) and a trend in response to 0.5 µg/mL collagen concentration (P=0.069, Table 4). There was, however, no significant treatment x time interaction for either agonist at either concentration (Table 4), but the iAUCs were significantly different (all P<0.05) demonstrating overall differences over the course of the study day. It was notable that there were dose-response relationships between agonist concentration and platelet aggregation. The inhibitory effect of the blackcurrant beverage on platelet aggregation was greater with the lower agonist concentration and the effect was particularly marked when ADP was used as agonist: platelet aggregation in response to10 µM ADP was 35.6 ± 6.0% at baseline, but decreased to 25.6 ± 5.4% 2 h after consumption of the blackcurrant beverage. In contrast, platelet aggregation in response to 100 µM ADP was 76.7 ± 1.1% at baseline and decreased marginally to 70.4 ± 2.3% 2 h post-

treatment. This effect was also apparent with collagen-induced platelet aggregation, albeit less marked.

4.3.5 Nitrite and nitrate

The blackcurrant beverage had no effect on nitrite or nitrate concentrations (data not shown), but it should be noted that the intra-assay CV for nitrite was very poor at 18.4%.

4.3.6 IL-8

There was a significant effect of treatment (P=0.0001), but no significant effect of time and no significant treatment x time interaction (P=0.264 and P=0.332, respectively; Figure 3). The change from baseline in IL-8 concentration was significantly higher over the course of the study day after consumption of placebo compared with intervention (Figure 3), this was also demonstrated by significant differences in iAUCs (P<0.05).

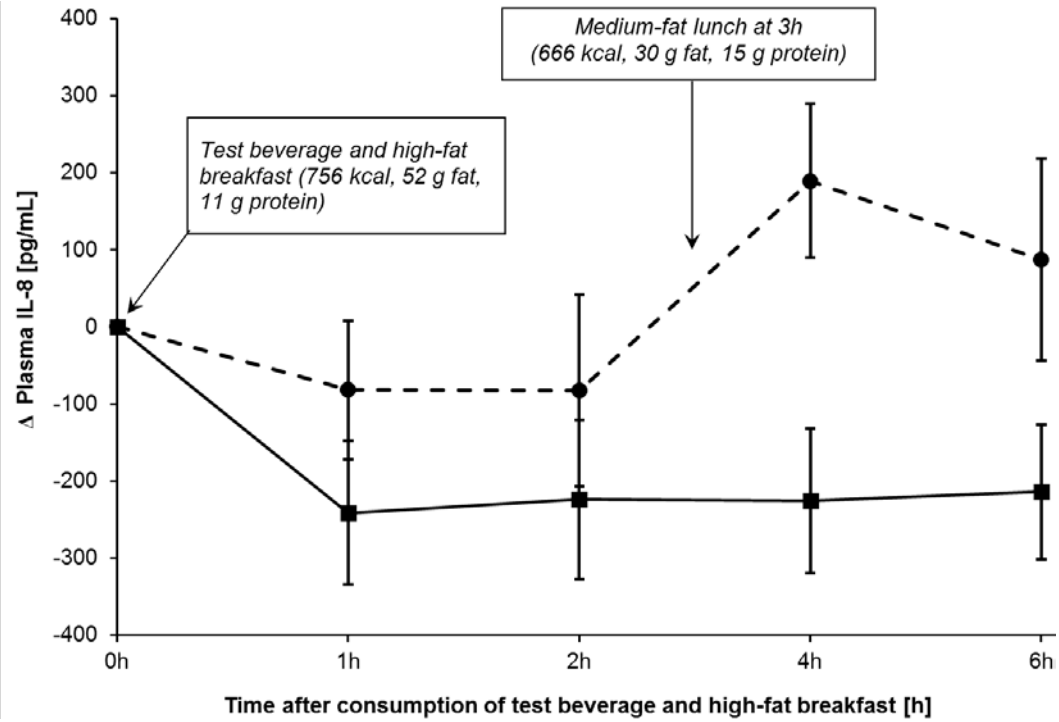

Figure 3 Change from baseline in plasma IL-8 concentrations (mean ± SEM) after consumption of the blackcurrant beverage containing 711 mg anthocyanins (solid line) or placebo (dashed line) together with a high-fat breakfast (50 g fat) (n = 23). *Volunteers consumed a medium-fat lunch (30 g fat, 15 g protein) 3 h after they had consumed the breakfast and test beverage. Differences from baseline in IL-8 concentrations were analyzed using a linear mixed model with treatment (2 treatments: blackcurrant compared to placebo beverage) and time (4 levels) as factors: the treatment effect was significant (P=0.0001), but there was no significant effect of time and no treatment x time interaction (P=0.264 and 0.332, respectively). Baseline IL-8 concentration was significantly higher on days where the blackcurrant beverage was consumed (paired t test, 2230 pg/mL vs 1913 pg/mL; P=0.008). IL-8, interleukin-8.*

4.3.7 Microparticles

The blackcurrant beverage had no effect on EMPs or PMPs (Table 3).

Table 3 Mean subject baseline and change from baseline for vascular function and inflammatory markers

		Change from baseline				*P* value	
	Baseline	1 h	2 h	4 h	6 h	Treatment	Treatment x time interaction
SBP (mm Hg)							
Placebo	113.1 ± 1.9	0.1 ± 0.9	-0.1 ± 1.0	3.1 ± 1.2	1.5 ± 1.0	0.015	NS
Blackcurrant	113.7 ± 2.3	-1.0 ± 1.0	-0.8 ± 1.3	0.9 ± 1.2	0.2 ± 1.3		
DBP (mm Hg)							
Placebo	65.2 ± 1.6	-3.8 ± 0.6	-2.9 ± 0.6	-3.3 ± 0.8	-2.7 ± 0.7	0.054	NS
Blackcurrant	65.5 ± 1.6	-4.5 ± 0.7	-3.7 ± 0.6	-4.0 ± 0.8	-3.6 ± 0.9		
DVP-SI							
Placebo	5.9 ± 0.7	nd	0.3 ± 0.2	0.2 ± 0.1	0.5 ± 0.1	NS	NS
Blackcurrant	5.9 ± 0.6	nd	0.4 ± 0.2	0.4 ± 0.2	0.7 ± 0.3		
DVP-RI							
Placebo	65.2 ± 1.2	nd	-5.5 ± 2.2	-10.0 ± 2.4	-6.8 ± 2.5	NS	NS
Blackcurrant	65.8 ± 1.1	nd	-6.6 ± 2.0	-12.2 ± 2.4	-9.7 ± 2.5		
PMPs (#/μL PPP)							
Placebo	1501 ± 103.9	nd	-171.1 ± 77.1	-139.8 ± 123.8	nd	NS	NS
Blackcurrant	1482 ± 102.2	nd	-118.5 ± 63.5	-171.9 ± 83.9	nd		
EMPs (#/μL PPP)							
Placebo	4536 ± 238.1	nd	-713.4 ± 214.5	-789.4 ± 202.0	nd	NS	NS
Blackcurrant	4457 ± 217.6	nd	-497.8 ± 182.4	-582.0 ± 178.9	nd		

n = 23. Data are means ± SEMs. Differences from baseline in study outcomes were analyzed using a linear mixed model for a 2-period crossover study. The model included volunteer as random factors and treatment, time and sequence as fixed effects. SBP, systolic blood pressure; DBP, diastolic blood pressure; NS, not significant; DVP, digital volume pulse; SI, stiffness index; RI, reflection index; nd, not determined; PMPs, platelet-derived microparticles; PPP, platelet-poor plasma; EMPs, endothelial-derived microparticles.

Table 4 Mean subject baseline and change from baseline for *ex vivo* ADP- and collagen-induced platelet aggregation

	Baseline		Change from baseline				*P* value	
			Placebo		Blackcurrant		Treatment	Treatment x time interaction
	Placebo	Blackcurrant	2 h	4 h	2 h	4 h		
ADP								
10 μM	27.5 ± 5.3	35.6 ± 6.0	1.6 ± 2.0	-3.4 ± 1.6	-10.0 ± 2.8	-11.0 ± 3.7	0.00003	NS
100 μM	74.1 ± 1.8	76.7 ± 1.1	2.0 ± 1.4	-2.5 ± 2.0	-6.3 ± 2.3	-5.6 ± 1.4	0.001	NS
Collagen								
0.5 μg/ml	44.6 ± 5.6	35.3 ± 5.9	-0.1 ± 2.3	-5.1 ± 3.2	-6.9 ± 2.0	-9.1 ± 4.2	0.069	NS
1 μg/ml	72.5 ± 2.5	74.7 ± 1.7	0.4 ± 2.2	-2.1 ± 1.9	-11.0 ± 3.2	-9.9 ± 3.4	0.0005	NS

n = 21. Data displayed are mean subject baseline and change from baseline. Platelet aggregation was assessed after 5 mins exposure to agonist. The extent of platelet aggregation was not different at baseline for either agonist at either concentration. Differences from baseline in study outcomes were analyzed using a linear mixed model for a 2-period crossover study. The model included volunteer as random factors and treatment, time and sequence as fixed effects.

4.4 Discussion

This RCT investigated the acute effects of an anthocyanin-rich blackcurrant beverage on FMD, platelet aggregation and a range of further CVD risk markers in response to a high-fat meal in a healthy, middle-aged population. There were significant improvements in vascular function, platelet aggregation, IL-8 and SBP, although the iAUC was not significantly different for the latter. The effects were observed with 711 mg anthocyanins, which corresponds to 120 mg fresh blackcurrants, an amount that is high but achievable through the diet. These findings may, at least in part, explain the cardio-protective properties associated with anthocyanins in epidemiological studies.

The RCT demonstrated, for the first time, a beneficial acute effect of blackcurrant-derived anthocyanins on FMD following a high-fat meal, and this supports previous investigations with 319-1,791 mg blueberry polyphenols in healthy men (9, 27), 694 mg açai polyphenols in overweight men (28) and 320 mg purified anthocyanins in hypercholesteraemic adults (29), although there was no effect of a boysenberry beverage containing 351 mg polyphenols on FMD in a small (and perhaps underpowered) study of six volunteers (30). In the current study, the magnitude of difference in FMD between both beverages over the course of the study day was 1.1% ($P_{treatment}<0.000001$, $P_{treatment*time\ interaction}=0.425$), which could be considered clinically relevant, if maintained chronically, as a 1% FMD increase is associated with a 13% reduced risk of cardiovascular events (31). The limited data to date suggests similar FMD benefits following 2-12 week consumption of anthocyanin-rich interventions (29, 30, 32), although a meta-analysis on the more widely studied chocolate/cocoa flavan-3-ols suggests FMD improvements to be less pronounced after chronic compared to acute intake (1.34 vs 3.19%) (33). In the current RCT, the maximum FMD improvement was observed at 2 h post-treatment with a 1.6% higher FMD compared to placebo, which is somewhat lower than effects

observed in RCTs providing anthocyanin-rich interventions without concomitant food intake, reporting maximum FMD improvements in the order of 2.5% (9, 27, 29). However, it is similar to a recent high-fat meal challenge study with açai polyphenols (28). Taken together, the evidence suggest that anthocyanins can also improve vascular function in postprandial conditions, albeit to a lesser degree. Nevertheless, since the majority of the day is spent in a postprandial state, and beverages are commonly consumed together with food, the study design employed in the current RCT is arguably more physiologically relevant than those where anthocyanins are delivered in the absence of food. A high-fat meal, has been reported to transiently impair several cardiometabolic functions, in particular vascular function (34-36), BP (37) and inflammation (22). However, the magnitude of meal-induced impairment of FMD was lower than previously reported (36), but there is evidence to suggest that this could partly be explained by the presence of proteins in the test meal (38), the predominance of butter fat in the test breakfast (39) and the volunteers' health status (40).

There is currently very limited information regarding the effect of anthocyanins on arterial stiffness. Although it has been shown to correlate with FMD (24), the current RCT reports that DVP-SI or DVP-RI were not acutely altered by blackcurrant anthocyanins. A similar lack of effect on arterial stiffness was observed following ingestion of 766 -1,791 mg blueberry polyphenols (9). However, a cross-sectional study reported an inverse association between higher anthocyanin intake and reduced arterial stiffness (5), and 8-week blueberry consumption has been reported to improve arterial stiffness (41), although acute improvements have also been reported with other other polyphenol classes (42, 43).

Data from this study indicate that an anthocyanin-rich blackcurrant beverage might beneficially ameliorate changes in BP that occur in response to a high-fat meal, while several previous RCTs report no acute effects following consumption of blueberry beverages (9, 27), a blueberry bun (27) or an açai smoothie (28). However, most studies tested lower amounts of anthocyanins

(range: 196-724 mg), indicating that high amounts might be required to modulate BP, but this requires confirmation.

To our knowledge, this is the first RCT investigating the acute effect of an anthocyanin-rich intervention on platelet function. Consumption of the blackcurrant beverage significantly inhibited *ex vivo* ADP- and collagen-induced platelet aggregation, which may have implications for thrombus formation. Only two previous studies examined the acute anti-platelet action of anthocyanin-rich products, but both focused on acute-on-chronic effects. In a small, uncontrolled study with ten volunteers, consumption of purple grape juice containing 1017 mg total polyphenols significantly inhibited collagen-induced platelet aggregation, but there were no effects with ADP or thrombin, indicating that only the collagen-induced signaling pathway was modulated (44). In an RCT with 35 healthy males, a smaller amount of 400 mg total polyphenols delivered as part of a wine grape extract capsule, had no significant effect on ADP-or collagen-induced platelet aggregation at 1.75 h post-treatment, but there was a trend for inhibited platelet aggregation after consumption of lunch and another wine grape extract capsule (45). However, the intervention products in both studies contained a significant proportion of other polyphenols and thus identification of the bioactive compounds is not possible. Chronic administration of anthocyanin-rich products has also been shown to improve platelet function in some (46-48), but not all studies (49-51). Direct comparisons between studies are virtually impossible as a number of different methods are used for platelet function assessment and important details regarding amount are often missing. The present study used the gold standard technique, *ex vivo* platelet aggregation in PRP, and this method has been shown to be associated with coronary heart disease mortality (11) and previous myocardial infarction (52).

There is evidence that improvement in vascular function by anthocyanins is mediated, at least in part, through modulation of endothelial nitric oxide metabolism. Inhibited neutrophil

NADPH oxidase activity (9) was reported in an acute blueberry RCT and elevated concentrations of cyclic guanosine monophosphate following supplementation with purified anthocyanins (29), but changes in superoxide production and nitric oxide bioavailability were not assessed. The present study failed to find any significant effects of the blackcurrant beverage on plasma nitrite or nitrate concentrations, although as 31% of samples were below the nitrite detection limit, sensitivity of the assay was an issue. Vasoactive substances other than nitric oxide might also be important determinants for FMD and might also be modulated by anthocyanins (53). For example, cocoa procyanidins (54) and the isoflavones genistein and daidzein (55) increased prostacyclin production, while quercetin and (–)-epicatechin decreased endothelin-1 production (56).

Previously it was demonstrated that improvements in FMD after 4 week consumption of a cocoa drink were paralleled by a decrease in the number of circulating EMPs, suggesting a causal link in preventing endothelial dysfunction (16). In contrast, the current study showed no significant effects on the number of circulating EMPs. However, the cocoa study was conducted in medicated patients with coronary artery disease, had a chronic design and investigated a different class of flavonoids. Interestingly, the potential importance of the volunteers' health status was suggested in another chronic cocoa study, where consumption of a daily cocoa bar reduced circulating numbers of EMPs in obese and overweight volunteers, who had initial higher values, but not in normal weight volunteers (17). To our knowledge, the present RCT is the first study to investigate the acute postprandial effect of an anthocyanin-rich intervention on circulating EMPs and PMPs and thus comparable data are lacking.

In conclusion, the present study demonstrates, for the first time, that an anthocyanin-rich blackcurrant beverage can beneficially ameliorate changes in vascular function and markers of CVD risk that occur in response to a high-fat meal. Improvements were observed in FMD, platelet function, IL-8 concentrations and SBP, although the iAUC was not significantly

different for the latter. The data suggest that anthocyanins can attenuate deleterious effects of a dietary fat challenge and could represent an effective tool for CVD prevention. Further studies are warranted to investigate underlying mechanisms of action and potential chronic effects.

4.5 Acknowledgments

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The authors' contributions were as follows: AMA, JPES, and PY designed the study; AMA, KA, DK, ISI, and RZ conducted the human study; GS and GC performed the polyphenol and metabolite analysis of plasma and urine samples, TB-M developed the methodology for the microparticle measurements, VS-K provided advice on the nitric oxide analyzer, HA performed the polyphenol analysis of the study beverages under supervision of PK, AMA analyzed data and performed statistical analyses; AMA and PY wrote the manuscript; PY had primary responsibility for final content, and all authors read and approved the final manuscript.

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Chapter 5 General discussion

ACNs are increasingly being recognized for their potential health benefits, including effects on the immune and cardiovascular system. Epidemiological evidence suggests that higher ACN consumption is linked with a lower risk of CVD (Cassidy 2013, Mink 2007, McCullough 2012) and a reduction in CVD risk markers (Jennings 2012, Cassidy 2011, Chun 2008). The development of CVD is recognized to include a strong inflammatory component and, thus, the initial stages of the PhD project explored the *in vitro* inflammatory and immune effects of ACNs with a view to using these to inform a subsequent human study. In particular, the aim of Chapter 2 was to characterize the *in vitro* effects of the strawberry-derived ACN Pg-3-glc and its metabolites on the production of selected pro- and anti-inflammatory cytokines in LPS-stimulated THP-1 cells and to compare the effects in human-derived monocytes vs macrophages. The aim of Chapter 3 was to characterize the effects of Pg-3-glc and its metabolites on the same of pro- and anti-inflammatory cytokines, but in a different cellular model, namely whole blood cultures. In addition, the effects on phagocytosis and oxidative burst activity were investigated in this Chapter. Given the limited effects reported in Chapter 2 and 3, the mounting evidence that phytochemicals modulate vascular function and CVD (Rodriguez-Mateos 2014a, Rodriguez-Mateos 2013) and the lack of human data for ACNs, it was considered important and timely to direct the human work towards vascular and platelet function. Thus, the aim of Chapter 5 was to address some of the key research gaps by conducting an RCT, which investigated the acute effects of an ACN-rich blackcurrant beverage on selected CVD risk markers in response to a high-fat meal in a healthy, middle-aged population.

The key findings of the PhD thesis are summarized below:

- There were modest effects on the production of pro- and anti-inflammatory cytokines in the human RCT and the cellular models. Importantly, the effects seem to differ according to the experimental model employed (THP-1 cells vs whole blood cultures vs human RCT).
- There was no effect of Pg-3-glc and three of its plasma metabolites on opsonization, engulfment and subsequent destruction of bacteria.
- The ACN-rich blackcurrant beverage beneficially ameliorated changes in selected markers of CVD risk (FMD, platelet function, systolic BP and IL-8 concentrations) in response to a high-fat meal relative to the placebo. The data suggest that ACNs may represent an effective tool for CVD prevention.

5.1 Effects of ACNs on the immune system

5.1.1 Value of *in vitro* screening studies

Cell culture models are a particularly useful tool in screening experiments, such as the ones reported in Chapters 2 & 3, where several compounds are being tested (often at various amounts), for example to examine potential structure-function and dose-response relationships. These cell experiments can be performed relatively inexpensively and rapidly and are often used to inform intervention studies that can only test a very limited number of compounds (and amounts). Immortalized cell lines, such as THP-1 cells (employed in Chapter 2), can undergo a very large number of cell divisions and are thus particularly useful for experiments requiring a large quantity of cells. As a further advantage, variability is minimized as experiments are performed in virtually uniform cells. Primary cells, for example, whole blood cultures (employed in Chapter 3), constitute a more physiological model because they retain their *in vivo* physiology. However, primary cells usually only have a limited life in culture and the quantity of available cells is usually limited. Both immortalized cell lines and primary cells are

a crucial tool for the study of cellular and molecular pathways. In the context of studying the effect of dietary factors on the immune and cardiovascular system, cell culture models are considered of crucial importance and are being used, for instance, to investigate effects on (i) inflammation and phagocytosis, for example using primary human monocytes or monocyte cell lines (e.g. THP-1 cell line) and whole blood cultures (ii) endothelial function, for example using isolated blood vessel rings or endothelial cell culture (e.g. HUVEC cell line), (iii) monocyte activation and chemotaxis, for example using primary human monocytes or monocyte cell lines (e.g. THP-1 cell line), (iv) foam cell formation, for example using human monocyte-derived macrophages or THP-1-derived macrophages or (v) cell-cell interactions in atherosclerosis pathogenesis using co-cultures of cells, for example endothelial and monocyte cells (Botham 2015).

5.1.2 Effects of ACNs on phagocytosis

The screening study reported in Chapter 3 examined, for the first time, the effects of the predominant strawberry ACN, Pg-3-glc, and three of its plasma metabolites (4-HBA, PCA and PGA) on phagocytosis and oxidative burst in a screening approach using whole blood cultures. There were no effects of any of the test compounds on phagocytosis of opsonized or non-opsonized *E. coli* or on oxidative burst activity. Thus, while it has previously been suggested that flavonoids might improve immune defenses, the current data suggest that changes in opsonization, engulfment or subsequent destruction of pathogens might not be the underlying mechanism of action, at least not in the case of Pg-3-glc and its metabolites. Alternative mechanisms of action could include, for example, improved natural killer cell activity, lymphocyte proliferation and cytokine production by lymphocytes. Whilst there is some suggestion that these mechanisms might be targeted by flavonoids, the evidence is not conclusive and requires further research.

5.1.3 Effects of ACNs on markers of inflammation

The effects of the strawberry-derived ACN Pg-3-glc and three of its plasma metabolites on LPS-stimulated cytokine production was investigated in THP-1 monocytes, THP-1 macrophages and whole blood cultures, with slightly different findings.

- In THP-1 monocytes, PCA and PGA inhibited IL-6 production. The effects in macrophages were slightly different. Whilst PGA inhibited IL-6 production by THP-1 derived macrophages, it also inhibited IL-10 production, which was not observed in the THP-1 monocytes.
- In the whole blood cultures, Pg-3-glc and PGA at physiologically relevant concentrations had anti-inflammatory properties; however, effects were modest, only observed at the lowest dose tested and limited to IL-10.
- In the human RCT, reductions were observed in IL-8 over the course of the study day; however, the intervention product did not contain Pg-3-glc, but other ACN classes.

Interpretation of the data is made difficult by the different nature of the employed models. Both cellular models utilized LPS as an inflammatory stimulus, while the human RCT measured the inflammatory response to a dietary fat challenge, which is a physiologically meaningful and relevant approach to be used within clinical human studies (Calder 2013). With regards to the cellular models, THP-1 cells were cultured in purified form under conditions different from their natural environment. In contrast, whole blood cultures contain all blood components and are therefore arguably more physiologically relevant. As such, it is possible that in the whole blood culture work, the activation of one cell type by the test compounds influenced another cell type, which could have contributed to discrepant findings (Crouvezier 2001).

5.1.4 Reflections on *in vitro* study design

Cell culture models provide a valuable addition to epidemiological and intervention studies, but in order to mimic physiological conditions their design requires knowledge of the

pharmacokinetics of treatment compounds. However, most studies exploring the effect of ACNs to modulate cytokine secretion were conducted using the unmetabolized parent ACNs rather than the physiologically more relevant metabolites and often testing supraphysiologically high concentrations (Roth 2014, Noratto 2011, Esposito 2014, Wang and Mazza 2002, Herath 2003, Jin 2006, Bognar 2013, Zhang 2010, Karlsen 2007, Karlsen 2010, Mossalayi 2014, Del Corno 2014, Zdarilova 2010). Chapter 2 and 3 examined the health properties of Pg-3-glc, the predominant ACN in strawberries. Pharmacokinetic studies have reported glucuronidated pelargonidin as the predominant metabolite (Mullen 2008, Sandhu 2016, Banaszewski 2013). However, there is ambiguity regarding the position of glucuronidation and scientific investigations are further hindered by the fact that glucuronidated pelargonidin compounds are currently commercially unavailable and hence could not be tested in the cell culture models reported in Chapter 2 and 3. 4-HBA and PCA have also been reported in plasma following strawberry consumption in low micromolar concentration (0.1-2 µM) (Russell 2009, Azzini 2010, Banaszewski 2013) and these were tested at the relevant concentration in the cellular work in Chapter 2 and 3. In addition, it is likely that PGA might appear in plasma following strawberry consumption, as it is a A-ring degradant, reported in plasma upon ACN consumption in low to high nanomolar concentration (20-600 nM) (de Ferrars 2014a, de Ferrars 2014b) and as such this was also tested at the relevant concentration in the cellular work in Chapter 2 and 3. Generally, the field of ACN pharmacokinetics has seen much progress in the last few years, especially with regards to Cy-3-glc. New evidence, which emerged in 2013 and 2014 (Czank 2013, de Ferrars 2014b, de Ferrars 2014a), demonstrated that its metabolism is far more complex than previously believed and as many as 24 degradants and metabolites were identified upon consumption of isotopically labelled Cy-3-glc. Importantly, the metabolites were found to appear at much higher concentrations in serum compared to the parent compounds and degradants. As such, these studies highlighted that, ideally, these newly

identified novel metabolites should be used in future cell and whole blood culture work concerned with studying Cy-3-glc bioactivity and this has been outlined by several experts (Kay 2015). It needs to be stressed in this regard, however, that progress in this area might be hindered by the fact that a large proportion of these metabolites are not commercially available yet (Table 2, Chapter 1). Whilst it is possible to specifically synthesize these compounds, it requires a cost- and time-intensive procedure that only produces small quantities. Thus, until the relevant compounds are commercially available, scientific progress might be hindered. As a result, there is very limited data regarding the biological activity of these newly identified metabolites. These were mainly conducted under supervision of Dr Colin Kay at the University of East Anglia, who was working with collaborators who synthesized the relevant metabolites (di Gesso 2015, Edwards 2015, Warner 2016, Amin 2015). The results suggest that ACN metabolites are bioactive, with an effect on haem oxygenase (Edwards 2015), cytokines and adhesion molecules (Amin 2015, di Gesso 2015). Also, very importantly, some of the studies indicated improved bioactivity of the metabolites as compared to their parent structures. It was, for example, reported that metabolites lowered LPS-induced TNF-α to a greater extent compared to the precursor compounds in THP-1 monocytes (di Gesso 2015). Similarly, experiments with other flavonoid classes reported differential bioactivities of the parent compound vs its metabolites. However, in the case of quercetin the metabolites were reported to have reduced bioactivity on cell adhesion molecule expression in endothelial cell line (Tribolo 2008) and smooth muscle cell line (Winterbone 2009) as well as endothelial function in rat aorta rings (Lodi 2009).

A further limitation relates to the fact that LPS was used at a relatively high concentration (1 µg/mL). LPS is a component of bacterial cell membranes and is frequently utilized as an inflammatory stimulus in cell culture experiments, for example to stimulate cytokine production as in the cellular work conducted as part of the present thesis. The concentration of

LPS in combination with the 24 h stimulation time were chosen to ensure stimulated production of all tested cytokines and to minimize variability. However, the LPS stimulation elicited very large increases in cytokine levels and it remains possible that the potent stimulation overruled potential protective effects of the tested phenolic compounds. However, it is important to bear in mind, that whilst a lower LPS concentration would potentially provide more scope for modulation and a better mimic of *in vivo* conditions, it would also be likely to increase variability. As mentioned before, LPS is frequently used as an inflammatory stimulus in experimental cell culture models, but it would also be of interest to investigate the effects using other inflammatory stimuli involved in atherosclerosis and CVD, for example oxidized LDL and soluble CD40L. In addition, the measurement of cytokine levels was conducted after a 24 h incubation period and it is unknown whether the effects would differ according to incubation period. The time-dependency could be investigated in time-course studies. Furthermore, the present work used a co-incubation approach of test compounds and LPS while other investigations pre-incubated cells with test compounds followed by later LPS-stimulation. It is unknown whether this would affect the result and could be a subject of further investigations. In addition, future work is required to elucidate the molecular mechanism of action for the observed effect. Possible approaches would be to test mRNA levels and effects on NF-κB or MAPK pathways. Future cellular work should also consider testing physiologically relevant mixtures of compounds as opposed to testing compounds in isolation. This seems of particular importance as recent evidence indicated that phenolic compounds might act additively/synergistically (Kay 2015).

In the present thesis, inflammatory status was assessed by measurement of a number of pro- and anti-inflammatory cytokines. However, it is important to bear in mind that inflammation is a complex process with multiple cells, mediators and pathways involved and thus there is also a significant number of markers that are associated with inflammation, namely

cytokines/chemokines (e.g. IL-1β, TNF-α, IL-6, IL-8, IL-10, MCP-1, RANTES), acute-phase proteins (e.g. CRP, von Willebrand factor, serum amyloid A, fibrinogen), adhesion molecules (VCAM-1, ICAM-1, E-selectin, P-selectin), adipokines (e.g. leptin and adiponectin) and blood cellular markers (leucocytes, neutrophils, T cells, eosinophils and monocytes). There is little consensus as to which markers are physiologically or pathophysiologically meaningful. Furthermore, all of these markers are characterized by significant between-individual variation and this is believed to be, at least in part, linked to several modifiers that affect the inflammatory response (age, body fatness, physical inactivity, sex, genetics, smoking, gut microbiota composition, diet, certain medication, emotional stress, pollution, viral infection and sleep behavior). A group of experts identified and reviewed a large number of candidate biomarkers, but concluded that no single marker can be pinpointed as a gold standard measure of inflammatory status in human nutritional studies. Instead, the best approach is believed to be the measurement of multiple markers and challenge models are believed to be particularly informative in this context (Calder 2013), such as the meal challenge model employed in the RCT reported in Chapter 4.

5.2 Effects of ACNs on the cardiovascular system

5.2.1 Effects of ACNs on vascular function

The results from the human intervention study reported in this thesis are the first time to demonstrate an acute effect of blackcurrant-derived ACNs on FMD following a high-fat meal. Whilst beneficial effects on FMD following acute consumption of ACN-rich interventions have previously been reported (Zhu 2011, Rodriguez-Mateos 2013, Rodriguez-Mateos 2014b, Alqurashi 2016) (summarized in Table 3, Chapter 1), the studies often applied a simpler study design, where an acute amount (usually low-fat and low-calorie) was provided in the absence

of food. Nevertheless, since the majority of the day is spent in a postprandial state, and beverages are commonly consumed together with food, the study design employed in the current RCT is arguably more physiologically relevant. Taken together, the novel evidence from the current RCTs suggests that ACNs can also improve vascular function in the physiologically relevant postprandial state. The RCT further provides evidence to suggest that the findings could be of importance on a population level. The observed magnitude of difference in FMD between both beverages over the course of the study day was 1.1% ($P_{treatment}$<0.000001, $P_{treatment*time\ interaction}$=0.425), which could be considered clinically relevant, if maintained chronically, as a 1% increase in FMD is associated with a 13% reduced risk of cardiovascular events (Inaba 2010).

With regards to the amount of ACNs, the intervention product in the current study contained 711 mg ACNs, which corresponds to 120 g fresh blackcurrants, an amount that is high but achievable through the diet. In previous studies, beneficial acute effects on FMD- or EndoPAT-assessed vascular function were observed with a 320 mg ACN dose (Zhu 2011), but not with a 51 mg dose (Jin 2011), and a dose-response relationship has been established with 129-310 mg blueberry-derived ACNs (319-766 mg total polyphenols) and FMD (Rodriguez-Mateos 2013). Future intervention studies should focus on further investigating the dose-response relationship, especially at lower concentrations, in order to determine the lowest ACN amount required to induce bioactivity and to evaluate whether there is a threshold amount, where further increases in amount do not translate to increased bioactivity.

To further progress knowledge on the effects of ACNs on vascular function, recently developed protocols to measure FMD in mice (Schuler 2014) and rats (Heiss 2008) could potentially provide a cost- and time-effective alternative or addition to human RCTs.

To provide an insight into potential mechanisms of action for the beneficial vascular effects reported in the current RCT, concentrations of nitrite and nitrate were determined in carefully processed plasma samples. Nitrate is considered an unreliable marker of eNOS activity (Bondonno 2016) and changes were not expected and not observed. With regards to nitrite, it was notable that the concentrations in collected plasma samples were extremely low and a major limitation of the analysis was that that 31% of samples were below the nitrite detection limit. In fact, the analytical challenges for nitrite assessment are well described in the literature and are the focus of method development work in the research community (Bondonno 2016). With the limited data obtained, there was no significant effect of the blackcurrant beverage on nitrite, but it is unknown whether this relates to the poor nitrite sensitivity of the assay or a true NO-independent mechanism of action. The limited clinical data that exist with ACN-rich interventions suggest modulatory activity on NO bioavailability. Inhibited neutrophil NADPH oxidase activity (Rodriguez-Mateos 2013) was reported in an acute blueberry RCT and elevated concentrations of cyclic guanosine monophosphate following supplementation with purified ACNs (Zhu 2011), but changes in superoxide production and NO bioavailability were not assessed. Similarly, in acute interventions with flavan-3-ol containing cocoa drinks in smokers (Heiss 2005) and non-smokers (Schroeter 2006), FMD improvements were paralleled by increases in the levels of circulating S- and N-nitros(yl)ated NO species. The two studies also demonstrated eNOS-dependency of these effects as the cocoa-induced increases in FMD and NO species were abolished by concomitant intravenous infusion of a NOS inhibitor. However, another study by the same research group, reports concomitant increases in FMD and circulating S- and N-nitros(yl)ated NO species upon a flavan-3-ol rich cocoa beverage, but no changes in plasma levels of nitrite or nitrate (Heiss 2003). More research is urgently required to unravel the mechanism of action for the beneficial FMD effect observed in the current study. Future RCTs could, for example, use the more robust reductive gas-phase chemiluminescence

method for assessment of NO status or examine neutrophil NADPH oxidase activity. Further studies could also include measurements of other vasoactive substances, given that previous research reports increased production of the vasodilator prostacyclin by cocoa procyanidins (Schramm 2001) and the isoflavones genistein and daidzein (Hermenegildo 2005) while decreased production of the vasoconstrictor endothelin-1 has been reported by quercetin and (–)-epicatechin (Loke 2008). In addition, animal experiments could also prove useful in unravelling the underlying mechanisms of action. For example, aortic rings could be isolated from rats fed an ACN-rich intervention vs control, and aortic rings examined for NOS activity, NO production, cyclic GMP content and superoxide production, similar to the approach taken by Benito (2002).

5.2.2 Effects of ACNs on platelet function

The RCT reported in Chapter 4, demonstrated, for the first time, anti-platelet effects of acute consumption of an ACN-rich blackcurrant beverage, which represents a highly novel and exciting finding. Research in this area is only just emerging and, as such, there is very limited information regarding the potential anti-platelet effects of ACNs and underlying molecular mechanisms. In fact, to our knowledge, only two previous studies have investigated the acute effects of ACN-rich interventions on platelet function (summarized in Table 3, Chapter 1). However, both studies were focused on acute-on-chronic effects and thus the results from the current RCT are the first to demonstrate a "real" acute effect of ACNs on platelet function.

Activated platelets have been proposed as CVD risk factor due to their contribution to the development and progression of endothelial inflammation and atherosclerosis and their role in thrombus formation (Ruggeri 2002, Linden and Jackson 2010, Vorchheimer and Becker 2006). Indeed, increased platelet activity has been observed in patients with CVD (Elwood 1991) and has been associated with increased CVD risk (Trip 1990, Thaulow 1991, Qayyum 2015).

Furthermore, antiplatelet medication has been shown to prevent cardiovascular events (Antithrombotic Trialists' Collaboration 2002, Antiplatelet Trialists' Collaboration 1994, Nemerovski 2012, Antithrombotic Trialists' Collaboration 2009, Kunutsor 2016). Thus the totality of evidence suggests cardiovascular benefit of suppressed platelet aggregation, but the benefit is currently nonquantifiable due to a lack of suitable data.

There were a number of methodological limitations of the platelet aggregation work in the RCT (Chapter 4), the most obvious being the significant between and within subject variabilities in platelet responses. Although study volunteers were asked to refrain from consuming drugs known to affect platelet function for 10 days before each study visit, wide variation in baseline measure of platelet function is very common, even with the most stringent protocols. In the case of the current study, the need to transport the samples to a neighboring building before analysis was unavoidable, but may have exacerbated the variation. A series of recommendations for handling samples for platelet analysis can be made based on recent studies examining the effect of a water-soluble tomato concentrate on platelet aggregation in human intervention studies (O'Kennedy 2006a, O'Kennedy 2006b, Lazarus 2004), which subsequently formed the supporting basis for an EFSA-approved health claim[1] (EFSA 2009). They include discarding blood samples showing evidence of platelet activation, temporarily withdrawing volunteers with an elevated inflammatory response and adding "normal platelet function" as an additional study inclusion criteria.

[1] Most studies were propriety and unpublished, and, as such, the suggestions are based on methodologies described in published protocols.

As research investigating the effect of ACNs on platelet function is only just emerging, several suggestions for future work can be made, for example:

- verify the present findings using other ACN classes (either delivered via a food or beverage or in the form of purified compounds) and wider population groups,
- investigate potential dose-response relationships and establish the lowest bioactive amount
- investigate the underlying mechanism of action. Due to time restrictions on study days and the exploratory nature of the platelet work, only two agonists were investigated at a limited number of concentrations in the RCT reported in Chapter 4, but future RCTs could investigate the response to a wider range of platelet agonists. As different agonists target different pathways in the aggregation cascade (Nardini 2007), the suggested approach would provide more insight into the potential mechanism of action and whether ACNs have a generic or selective action in the signaling cascade of platelet aggregation. For example, supplementation of coffee extract to rabbits has an inhibitory effect on ADP- and AA-stimulated platelet aggregation, but no effect on collagen-induced platelet aggregation (Bydlowski 1987). More information is needed to unravel the specific molecular processes that are being modulated.

5.2.3 Effects of ACNs on circulating EMPs and PMPs

To our knowledge, the RCT reported in Chapter 4 is the first study to investigate the acute postprandial effect of an ACN-rich intervention on circulating EMPs and PMPs, although no significant effects were observed. Because of the novelty, comparable data on ACN-rich interventions are lacking, but it was previously demonstrated that improvements in FMD after 4-week consumption of a cocoa drink were paralleled by a decrease in the number of circulating EMPs, suggesting a causal link in preventing endothelial dysfunction (Horn 2014). However, the cocoa study was conducted in medicated patients with coronary artery disease, had a chronic design and investigated a different class of flavonoids. Interestingly, the potential importance of the volunteers' health status was suggested in another chronic cocoa study, where

consumption of a daily cocoa bar reduced circulating numbers of EMPs in obese and overweight volunteers, who had initial higher values, but not in normal weight volunteers (McFarlin 2015).

A limitation of the MP work in the RCT reported in Chapter 4 relates to the use of Megamix beads for flow-cytometry-based sizing of MPs. Megamix beads are standardized reference beads of known size and they were used in the present RCT to define a forward scatter MP size gate to in order to distinguish MPs from other particles, in particular larger platelets. However, the Megamix beads have recently been criticized with regards to their usefulness to calibrate size gates. Megamix beads consist of polystyrene and have been reported to scatter more light compared to equivalently sized biological cells and MPs. As a result, their actual size is overestimated, potentially causing erroneous size calibration (Chandler 2011, van der Pol 2014 and personal communication with Dr Dionne Tannetta, University of Reading). As a result, it is likely that the defined MP gate in the current study contained not only MPs, but also platelets (personal communication with Dr Dionne Tannetta, University of Reading). To overcome this difficulty, it has been proposed to use silica beads as their refractive index is closer to biological material, or develop a method of accounting for the difference in refractive index between synthetic beads and biological material (Chandler 2011, van der Pol 2014 and personal communication with Dr Dionne Tannetta, University of Reading). This highlights the fact that this is as yet an emerging area and the methodology is continually being refined. Most of the papers currently being published are still reporting use of Megamix beads and it will likely take some time before this changes, particularly as the companies which market the beads do so on the basis of incorrect sizing information. It is true to say that there is also much inconsistency regarding antibody selection and the use of a range of techniques for MP analysis (transmission electron microscopy, flow cytometry, nanoparticle tracking analysis and resistive pulse sensing) and a gold standard method has not been defined yet (van der Pol 2014).

The health impact of elevated levels of circulating MPs is not without controversy. Supporting evidence for a role of PMPs and EMPs in CVD pathogenesis comes from studies demonstrating that increased levels of circulating PMPs and EMPs are found in patients with CVD risk factors, such as obesity (La Vignera 2012, McFarlin 2015) and hypertension (Mallat 2000) and they are increased in patients with acute coronary syndromes (Mallat 2000) and after acute myocardial infarction (Boulanger 2006). In contrast, it has been suggested that some MP can have beneficial properties through acting on promotion of endothelial progenitor cell differentiation, increasing NO bioavailability and inducing angiogenesis. However, research into this area is only just emerging (Martinez 2011).

5.2.4 Reflections on intervention study design

A major strength of the RCT reported in Chapter 4 is the physiologically relevant study design. Acute effects of the ACN-rich intervention were investigated following consumption of meals, mimicking everyday life conditions, where the human body is in the postprandial state for the majority of the day. Thus, the study design employed in the current RCT is arguably more physiologically relevant than those where ACNs are delivered in the absence of food.

In the current study, considerable attention was devoted to matching the intervention and placebo beverage. For example, matching was ensured for vitamin C, as it is known to improve vascular function (Ashor 2014). However, it was not possible to accurately match all of the micronutrients, which is a potential limitation. This is, in fact, common in polyphenol research applying a "whole food" approach. Most polyphenol-rich intervention vehicles provide a number of different polyphenols (stemming from different polyphenol subclasses) and in addition a number of other potentially bioactive compounds. Adequate placebo-matching is by nature limited. To overcome this difficulty, a few intervention studies have employed purified polyphenols in order to better evaluate the bioactivity of specific compounds (Schroeter 2006,

Dower 2015, Widlansky 2007). However, using a whole food approach is arguably more physiological as ACNs are rarely consumed in isolation and, in addition, evidence suggests that synergies exist between different phenolic compounds (Kay 2015).

The focus of the current study was on acute effects over a 6 hr post-treatment period and the results point to novel and potentially exciting clinical significance. Complementary chronic studies would allow further development of this interpretation. In terms of study design, it would have been desirable to be able to monitor effects over a longer timeframe (for example 24 hrs) given recent pharmacokinetic data on labelled Cy-3-glc, which revealed that its main phenolic metabolites (hippuric acid and ferulic acid) reach serum peak concentration at approximately 16 and 8 h respectively (de Ferrars 2014b). This was considered during study design, but deemed not possible due to logistical challenges and a lack of ability to monitor volunteers over longer time periods. It is likely for the same reason, that such data is currently not available for cardiovascular outcomes. Further pharmacokinetic data on labelled ACNs, possibly investigating additional ACN classes, would be important in future to obtain critical information to inform the design of *in vitro* experiments and human RCTs.

It is further important to consider whether the amount of ACNs employed is achievable if it is to relate to public health recommendations. In the study described in this thesis, the 200 mL portion of the blackcurrant beverage contained 711 mg ACNs (744 mg polyphenols). On a food level, this corresponds to approximately one and a half portions (120 g) of fresh blackcurrants (Neveu 2010), which is a rather large amount to consume in one sitting, albeit achievable. It further exceeds the amount found in 13 commercially available blackcurrant juices, which were shown to contain ACN contents ranging from 0.28 to 98.4 mg/200 mL (Nielsen 2003). The blackcurrant beverage used in this RCT was prepared by dissolving spray-dried blackcurrant powder in 200 mL of water. The blackcurrant powder was purchased from BerryPharma (Leichligen, Germany, product 70140015, batch L 14IV04342) who apply a special processing

technique to enrich the ACN content of the powder, thus "squeezing" 711 mg ACNs into 5.7 g of concentrated powder. As a 200 mL beverage, the 711 mg ACNs are an easily achievable dietary amount. This higher amount was also chosen for the RCT given beneficial health effects would commercially support processing techniques to enrich juices with ACNs and market them as such. From a scientific perspective, the current study would support this.

5.2.5 Reflections on dietary guidelines and commercial aspects

The results provide further support to dietary recommendations for a high fruit and vegetable consumption. This is of particular importance, given fruit and vegetable intake is well below dietary recommendations in many countries (Department of Health 2016, Guenther 2006, Kimmons 2009, Nicklett and Kadell 2013, Myint 2007, Yngve 2005).

The beneficial health effects of highly concentrated ACNs can be combined with recent research on methods to protect ACNs from degrading (Burin 2011) and used by the food industry for innovative functional food products, e.g. the blackcurrant powder could be added to beverages, yoghurt, porridge or used in dietary supplements. In order to be able to communicate these benefits to consumers, further effort is required to comply with current regulatory requirements. Under the Nutrition and Health Claims Regulation that came into force in 2007, for a health claim to be permitted on a food/drink/dietary supplement a dossier with robust RCTs needs to be submitted to EFSA, receive their positive opinion and be authorized by the European Commission (Buttriss 2015a, Buttriss 2015b, European Commission 2007). There is an example for a polyphenol-rich product that has successfully completed this process. A health claim has been approved for the link between cocoa flavanol intake and improved vascular function (the claim reads "Cocoa flavanols help maintain endothelium-dependent vasodilation, which contributes to normal blood flow") (EFSA Panel on Dietetic Products 2012). As putting together a dossier requires substantial resources, a new

EU project named "*BACCHUS*" aims to develop tools, resources and scientific evidence to support health claim efforts (http://www.bacchus-fp7.eu/, accessed 25 November 2016).

5.3 Future perspectives

This thesis sets out convincing evidence of acute effects of an ACN-rich blackcurrant beverage on FMD, platelet aggregation, SBP and IL-8, but there are a number of key research gaps to be addressed in the future. Firstly, it would be important for future studies to further investigate potential dose-response relationships, especially at lower concentrations, in order to determine the lowest ACN amount required to induce bioactivity and to evaluate whether there is a threshold amount, where further increases in amount do not translate to increased bioactivity. Furthermore, the molecular mechanisms underlying the observed beneficial effects on FMD and platelet function are largely unknown. Further research should explore potential NO-dependent and -independent mechanisms of action, employing robust analytical techniques.

To test for potential structure-function- and dose-response-relationships, the present thesis employed *in vitro* screening models investigating the effect of the strawberry-derived ACN Pg-3-glc and three of its physiologically relevant plasma metabolites on selected markers of inflammation and immunity. The whole blood culture work demonstrated that there was no effect of any of the tested compounds on phagocytosis or oxidative burst activity, thus suggesting that subsequent studies should explore other immunomodulatory effects (for example natural killer cell activity or lymphocyte proliferation).

5.4 Concluding remarks

The thesis has significantly contributed to the emerging evidence that ACNs confer promising cardioprotective potential, providing further support to dietary recommendations encouraging

fruit and vegetable consumption. The novel work reported in this PhD thesis further suggests that the beneficial vascular effects of ACNs are conferred via a number of different mechanisms. In particular, by improving vascular function, platelet aggregation, BP and the inflammatory response. Key research efforts in the future should focus on unravelling the underlying mechanism of action for the FMD and platelet effect and investigate dose-response relationships. Finally, to ultimately prove a cause and effect relationship between ACNs and CVD, long-term RCTs with hard clinical endpoints would be desirable.

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Communication

Pelargonidin-3-O-glucoside and its metabolites have modest anti-inflammatory effects in human whole blood cultures

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ABSTRACT

This study hypothesized that the predominant strawberry anthocyanin, pelargonidin-3-O-glucoside (Pg-3-glc), and 3 of its plasma metabolites (4-hydroxybenzoic acid, protocatechuic acid, and phloroglucinaldehyde [PGA]) would affect phagocytosis, oxidative burst, and the production of selected pro- and anti-inflammatory cytokines in a whole blood culture model. For the assessment of phagocytosis and oxidative burst activity of monocytes and neutrophils, whole blood was preincubated in the presence or absence of the test compounds at concentrations up to 5 μmol/L, followed by analysis of phagocytic and oxidative burst activity using commercially available test kits. For the cytokine analysis, diluted whole blood was stimulated with lipopolysaccharide in the presence or absence of the test compounds at concentrations up to 5 μmol/L. Concentrations of selected cytokines (tumor necrosis factor-α, interleukin [IL]-1β, IL-6, IL-8, and IL-10) were determined using a cytometric bead array kit. There were no effects of any of the test compounds on phagocytosis of opsonized or nonopsonized *Escherichia coli* or on oxidative burst activity. Pg-3-glc and PGA at 0.08 μmol/L increased the concentration of IL-10 ($P < .01$ and $P < .001$, respectively), but there was no effect on tumor necrosis factor-α, IL-1β, IL-6, and IL-8, and there were no effects of the other compounds. In conclusion, this study demonstrated a lack of effect of these compounds on the opsonization, engulfment, and subsequent destruction of bacteria. Pg-3-glc and PGA, at physiologically relevant concentrations, had anti-inflammatory properties; however, effects were modest, only observed at the lowest dose tested and limited to IL-10.

1. Introduction

Anthocyanins are polyphenols which are abundant in berry fruits and which may convey health benefits to humans, including cardiovascular disease prevention, obesity control, alleviation of diabetes, improvement of vision and memory, and increased immune defenses [1,2]. An anthocyanin-rich elderberry extract was demonstrated to exert antimicrobial and antiviral activity in vitro toward human pathogenic respiratory bacteria and influenza viruses, although mechanisms were unclear [3]. Leukocytes play a crucial role in pathogen defense [4], and several leukocyte functions have

Abbreviations: 4-HBA, 4-hydroxybenzoic acid; FCS, fetal calf serum; IL, interleukin; LPS, lipopolysaccharide; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; TNF-α, tumor necrosis factor-α.

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been reported to be influenced by flavonoids, although some of the data are conflicting. Blueberries [5], an anthocyanin-rich juice [6], and purple sweet potato leaves [7] were reported to increase numbers and activity of natural killer cells [5], but there was no effect of red wine, dealcoholized red wine, or red grape juice [8,9]. Similarly, lymphocyte proliferation and interleukin (IL)-2 secretion by activated lymphocyte were increased upon consumption of an anthocyanin-rich juice [6] and purple sweet potato leaves [7] but not affected by red wine, dealcoholized red wine, or red grape juice [8,9]. Red wine, dealcoholized red wine, or red grape juice also had no effect on phagocytosis by neutrophils and monocytes [8,9]. In an animal model, red wine anthocyanins increased phagocytic activity at 25 and 50 mg/kg body weight but decreased phagocytic activity at higher doses [10]. In mice, pyogallol-type green tea polyphenols increased phagocytic activity in vitro [11], and a polyphenol-rich cereal fraction increased phagocytic activity and increased production of reactive oxygen species and superoxide anion [12], but a number of anthocyanin- and flavonoid-rich fruits were reported to diminish reactive oxygen species production [13]. There are a lack of evidence from human studies and limited and conflicting data regarding the influence of flavonoids on the phagocytic process.

Several cell culture studies have explored the effect of anthocyanins to modulate cytokine production and other parameters of immune function, but most were conducted using the unmetabolized parent anthocyanins, often at high doses [3,10,13-26], which may not be physiologically relevant. Strawberries constitute a popular fruit, and they are particularly rich in anthocyanins, predominantly pelargonidin-3-O-glucoside (Pg-3-glc) [27]. Glucuronidated pelargonidin has been reported as the predominant metabolite in 3 pharmacokinetic studies [28-30], but there is ambiguity regarding the position of glucuronidation, and glucuronidated pelargonidin compounds are currently commercially unavailable and hence cannot be tested in cell culture models. 4-Hydroxybenzoic acid (4-HBA) and protocatechuic acid (PCA) have also been reported in plasma following strawberry consumption in low–micromole per liter concentration (0.1–2 μmol/L) [30-32]. In addition, it is likely that phloroglucinaldehyde (PGA) might appear in plasma following strawberry consumption, as it is an A-ring degradant, reported in plasma upon anthocyanin consumption in low– to high–nanomole per liter concentration (20-600 nmol/L) [33,34]. It is important to note in this context that 4-HBA, PCA, and PGA are not pelargonidin-specific metabolites. Their presence in plasma has been reported following ingestion of other flavonoids, and they are also naturally present in several other dietary sources. However, there are very little data on the effects of these physiologically relevant compounds. Furthermore, most in vitro work has been conducted using cell lines, but whole blood cultures more closely represent physiological conditions [35,36]. This study therefore characterized the effect of the parent anthocyanin Pg-3-glc and 3 physiologically relevant plasma metabolites on phagocytosis, oxidative burst, and the production of selected pro- and anti-inflammatory cytokines (tumor necrosis factor-α [TNF-α], IL-1β, IL-6, IL-8, and IL-10) in a whole blood culture model to test our hypothesis that modulations would be observed.

2. Methods and materials

2.1. Subjects

Ten healthy volunteers (8 women and 2 men) were recruited for this pilot study. Inclusion criteria included the following: 40-65 years old; good general health; and absence of diabetes, cancer, liver cirrhosis, asplenia, other acquired or congenial immunodeficiency, HIV, or any kind of inflammatory, autoimmune, or connective tissue disease. The exclusion criteria were use of anti-inflammatory or immunomodulating medication, use of antibiotics within 3 months, vaccination within 3 months, participation in another drug or nutritional research study within 3 months, and alcoholism or drug misuse. Subjects were asked to follow a low-flavonoid diet for 24 hours prior to the blood sample collection. Volunteers arrived following a 12-hour fast to the Hugh Sinclair Unit of Human Nutrition of the University of Reading, and blood was collected into sodium heparin vacutainer tubes (Greiner Bio-One Ltd, Gloucestershire, UK). Written informed consent was obtained from all subjects. The work was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the University of Reading Research Ethics Committee (Project reference 10/05).

2.2. Materials

Pg-3-glc was purchased from Extrasynthese (Genay, France). PCA (3,4-dihydroxybenzoic acid), 4-HBA, PGA (2,4,6-trihydroxybenzaldehyde), lipopolysaccharides from *Escherichia coli* (LPS), methanol, and formic acid were purchased from Sigma-Aldrich (Dorset, United Kingdom). RPMI 1640 culture medium, fetal calf serum (FCS), and antibiotics (penicillin and streptomycin) were purchased from Lonza (Basel, Switzerland). The cytometric bead array kit to analyze cytokine concentrations was purchased from BD Biosciences (Oxford, United Kingdom). Phagoburst and Phagotest kits as well as the opsonized *E coli* bacteria were manufactured by Glycotope Biotechnology (Heidelberg, Germany). Pg-3-glc, PCA, 4-HBA, and PGA were dissolved in acidified methanol (2% formic acid) to a concentration of 10 mmol/L and stored at –70°C and away from light. Further dilutions of the test compounds were prepared freshly on each study day by dilution in RPMI medium with added FCS and antibiotics. RPMI culture medium was stored at 4°C. FCS and antibiotics were defrosted upon delivery, aliquoted, and stored at –20°C until use.

2.3. Whole blood culture for phagocytosis and oxidative burst capacity

Heparinized whole blood samples were preincubated with the test compounds (Pg-3-glc, PCA, 4-HBA, PGA, or RPMI 1640 medium as control) at 4 different concentrations (0.08, 0.31, 1.25, and 5 μmol/L) at 37°C for 4 hours in 15 × 75–mm tubes.

2.4. Whole blood culture for cytokine analysis

Heparinized whole blood was diluted 6:10 with RPMI 1640 medium supplemented with FCS and antibiotics. The diluted

blood (1 mL/well) was placed into 24-well tissue culture plates. Working solutions of the test compounds were added to provide final concentrations of 0.08, 0.31, 1.25, and 5 μmol/L. Respective volumes of RPMI 1640 medium were added to control cultures (no polyphenols). LPS (1-μg/mL final concentration) was added to stimulate cytokine production. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. At the end of the culture period, plates were centrifuged at 260*g* for 5 minutes. Culture supernatants were collected and stored in aliquots at –20°C until analysis.

2.5. *Measurement of leukocyte phagocytosis and oxidative burst capacity*

Phagocytic and oxidative burst activities of monocytes and neutrophils were analyzed using commercially available test kits (Phagotest and Phagoburst) following the instructions of the manufacturer. The percentage of neutrophils or monocytes engaged in phagocytosis of *E coli* bacteria (opsonized and nonopsonized) and oxidative burst activity and the mean fluorescence intensity were acquired on a BD FACS Canto II flow cytometer. Data were analyzed using DIVA software.

2.6. *Measurement of cytokine concentrations*

Concentrations of TNF-α, IL-1β, IL-6, IL-8, and IL-10 in the culture supernatants were measured using a cytometric bead array kit from BD Biosciences (Oxford, United Kingdom) according to the manufacturer's instructions. The intensity of the fluorescence signal was acquired on a BD FACS Canto II flow cytometer, and data were analyzed using the BD FCAP Array v3 software. Limits of detection of the cytokine assays

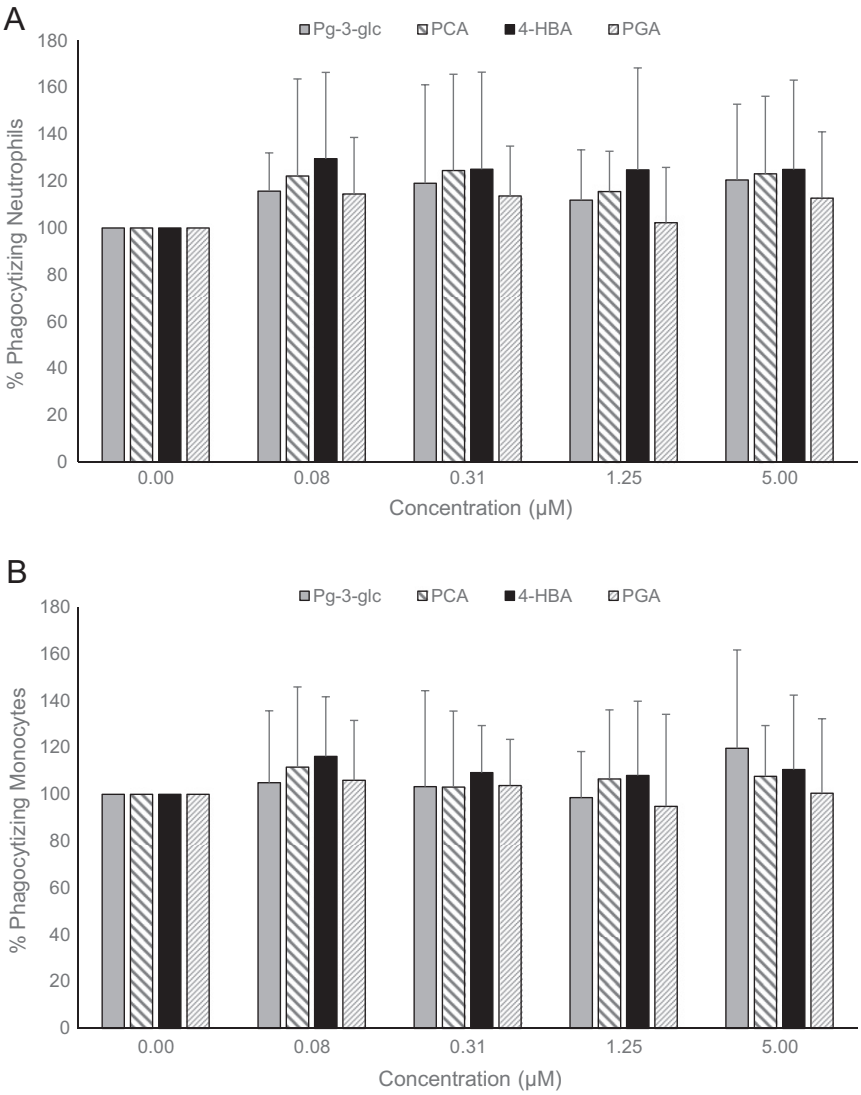

Fig. 1 – Effect of Pg-3-glc, PCA, 4-HBA, and PGA on phagocytic activity of nonopsonized *E coli* bacteria in human neutrophils (A) and monocytes (B). Human whole blood (n = 10) was treated with Pg-3-glc, PCA, 4-HBA, PGA, or vehicle control at concentrations of 0-5 μmol/L, for 4 hours at 37°C. Phagocytic activity was analyzed using the Phagotest test kit. Results are expressed as percentage of phagocytic activity vs control (no polyphenols). Data are represented as the means ± SD. Data were analyzed by 1-way ANOVA, and a lowered P < .01 was considered significant to account for multiple comparisons. There were no statistically significant changes.

are 0.13 pg/mL (IL-10), 1.2 pg/mL (TNF-α and IL-8), 1.6 pg/mL (IL-6), and 2.3 pg/mL (IL-1β).

2.7. Statistical analyses

Results are expressed as percentage of phagocytic activity/oxidative burst activity/cytokine production vs control (no polyphenols) and shown as means with their standard deviations (SD). One-way analysis of variance (ANOVA) was performed, followed by Dunnett as post hoc analysis vs control group where appropriate. Statistical analysis was performed using SPSS 21 (IBM Corporation, Armonk, NY, USA), and a lowered $P < .01$ was considered significant to account for multiple comparisons.

3. Results

3.1. Effects of Pg-3-glc, PCA, 4-HBA, and PGA on phagocytic and oxidative burst activity

None of the test compounds significantly affected the overall percentage of neutrophils or monocytes engaged in phagocytosis of opsonized or nonopsonized *E coli* bacteria and their oxidative burst activity (Figs. 1-3). A high degree of intersubject variability was notable. Furthermore, there were no significant effects of any of the test compounds on the mean fluorescence intensity, which indicates degree of phagocytic/oxidative burst activity (data not shown).

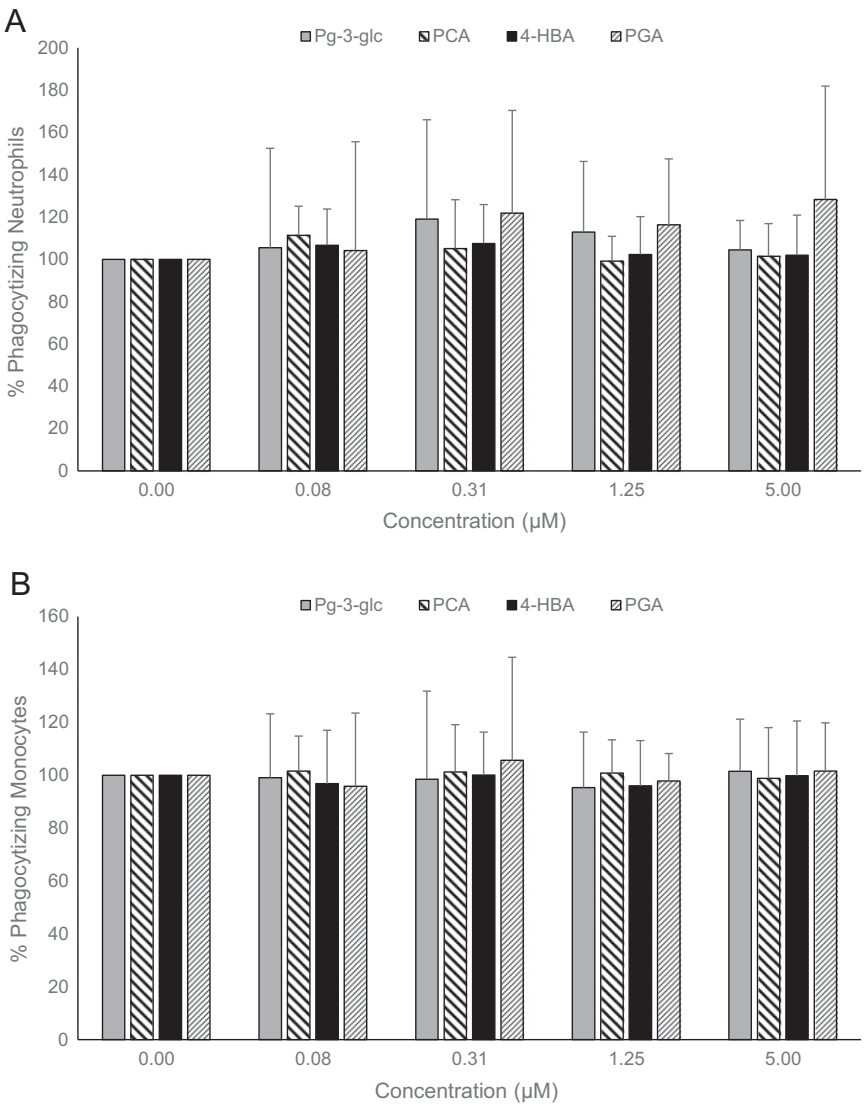

Fig. 2 – Effect of Pg-3-glc, PCA, 4-HBA, and PGA on phagocytic activity of opsonized *E coli* bacteria in human neutrophils (A) and monocytes (B). Human whole blood (n = 10) was treated with Pg-3-glc, PCA, 4-HBA, PGA, or vehicle control at concentrations of 0-5 μmol/L, for 4 hours at 37°C. Phagocytic activity was analyzed using the Phagotest test kit. Results are expressed as percentage of phagocytic activity vs control (no polyphenols). Data are represented as the means ± SD. Data were analyzed by 1-way ANOVA, and a lowered $P < .01$ was considered significant to account for multiple comparisons. There were no statistically significant changes.

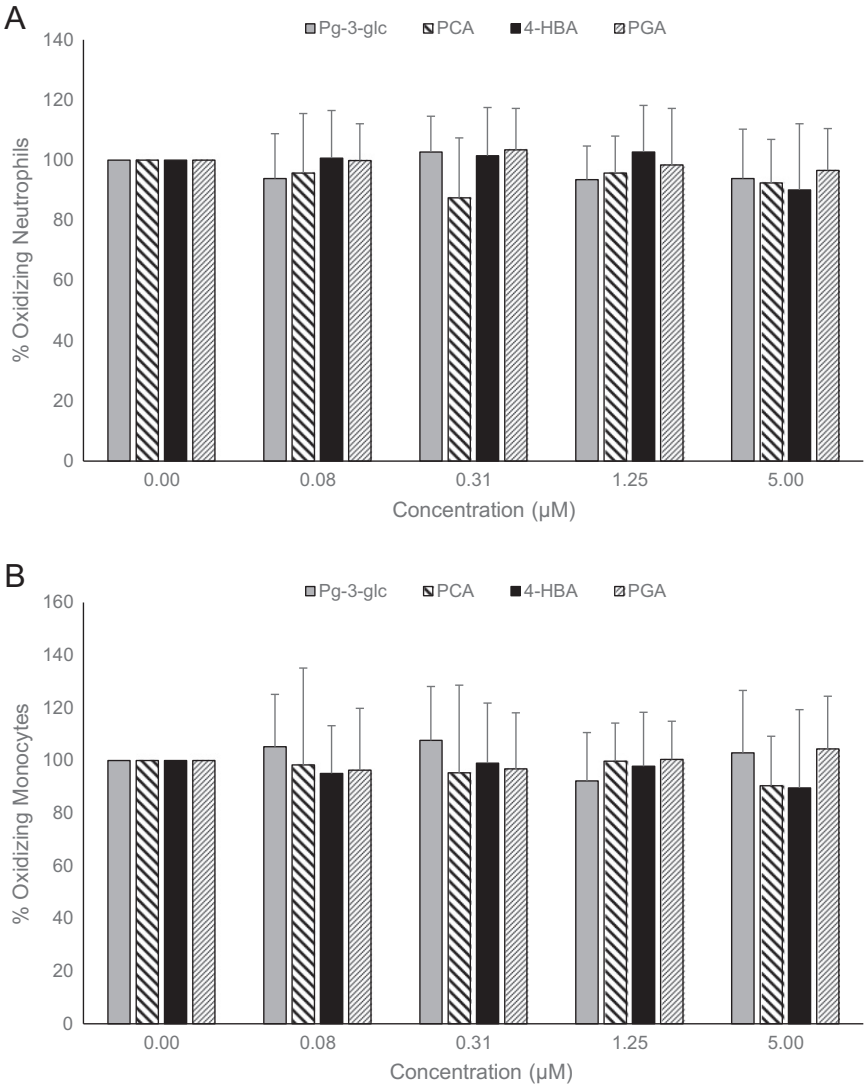

Fig. 3 – Effect of Pg-3-glc, PCA, 4-HBA, and PGA on oxidative burst activity in human neutrophils (A) and monocytes (B). Human whole blood (n = 10) was treated with Pg-3-glc, PCA, 4-HBA, PGA, or vehicle control at concentrations of 0-5 μmol/L, for 4 hours at 37°C. Oxidative burst activity was analyzed using the Phagoburst test kit. Results are expressed as percentage of oxidative burst activity vs control (no polyphenols). Data are represented as the means ± SD. Data were analyzed by 1-way ANOVA, and a lowered $P < .01$ was considered significant to account for multiple comparisons. There were no statistically significant changes.

3.2. *Effects of Pg-3-glc, PCA, 4-HBA, and PGA on cytokine production*

Stimulation with LPS increased IL-1β production 11-fold, TNF-α production 9-fold, IL-6 production 2-fold, IL-8 production 1-fold, and IL-10 production 5-fold. Pg-3-glc and PGA at the lowest dose tested (0.08 μmol/L) significantly increased IL-10 production compared with the control cultures ($P < .01$ and $P < .001$, respectively; Table 1). There was no significant effect of any tested compound, at concentrations up to 5 μmol/L, on the production of IL-1β, TNF-α, IL-6, or IL-8 by human whole blood cultures (Table 1).

4. Discussion

The current study investigated, for the first time, the effects of the strawberry-derived anthocyanin Pg-3-glc and 3 of its physiologically relevant plasma metabolites on phagocytosis, oxidative burst, and the production of selected pro- and anti-inflammatory cytokines by whole blood cultures, and our hypothesis was only partially supported by the results. There were no effects of any of the test compounds on phagocytosis or on oxidative burst activity. In agreement with these data, some human intervention studies report no significant effects of anthocyanin-rich red wine, red grape juice [8,9], or quercetin [37-40] on phagocytic ability of monocytes and neutrophils. However, higher doses of red wine anthocyanins did increase phagocytic activity in mice [10], suggesting that the outcomes of animal and human studies may differ and that dose might be important.

Once pathogens are engulfed by phagocytes (phagocytosis), they are destroyed in part by the production of reactive oxygen metabolites in a process termed *oxidative burst* [4]. In the current study, contrary to our hypothesis, there were no

Table 1 – Effect of Pg-3-glc, PCA, 4-HBA, and PGA on IL-1β, TNF-α, IL-6, IL-8, and IL-10 production in human whole blood cultures

Cytokine production (% of control)	Pg-3-glc				PCA				4-HBA				PGA			
	0 μmol/L	0.08 μmol/L	0.31 μmol/L	1.25 μmol/L	5.00 μmol/L	0 μmol/L	0.08 μmol/L	0.31 μmol/L	1.25 μmol/L	5.00 μmol/L	0 μmol/L	0.08 μmol/L	0.31 μmol/L	1.25 μmol/L	5.00 μmol/L	0 μmol/L	0.08 μmol/L	0.31 μmol/L	1.25 μmol/L	5.00 μmol/L
IL-1β	100	95.8 ± 11.2	96.6 ± 11.1	93.0 ± 10.4	90.3 ± 13.8	100	99.0 ± 7.6	99.4 ± 7.1	99.8 ± 10.7	96.8 ± 9.8	100	91.2 ± 7.9	92.9 ± 7.6	90.8 ± 7.6	92.4 ± 17.0	100	93.4 ± 14.6	94.4 ± 8.7	98.4 ± 11.2	108.4 ± 13.7
TNF-α	100	100.2 ± 26.1	96.9 ± 14.6	96.1 ± 12.0	96.6 ± 12.0	100	108.0 ± 13.5	95.0 ± 16.1	95.3 ± 20.3	97.7 ± 16.7	100	101.0 ± 8.8	99.6 ± 8.8	98.4 ± 9.2	103.8 ± 22.2	100	95.6 ± 26.8	102.0 ± 19.1	100.2 ± 14.9	98.4 ± 13.3
IL-6	100	98.5 ± 5.4	93.9 ± 6.7	94.7 ± 9.1	93.5 ± 9.2	100	102.0 ± 10.6	94.8 ± 9.8	94.6 ± 10.5	95.3 ± 7.0	100	96.0 ± 7.0	94.4 ± 7.2	94.7 ± 9.2	98.7 ± 8.7	100	100.6 ± 11.6	96.0 ± 9.7	97.3 ± 9.4	93.2 ± 8.6
IL-8	100	106.2± 14.5	93.2 ± 10.4	89.8± 10.7	88.9 ± 11.0	100	106.0 ± 23.6	89.5 ± 15.0	89.8 ± 14.7	90.3 ± 13.5	100	108.5 ± 21.0	98.1 ± 15.6	95.9 ± 14.9	95.3 ± 12.5	100	109.3 ± 13.8	96.4 ± 10.2	94.9 ± 11.9	103.8 ± 15.4
IL-10	100[a]	118.0 ± 22.6[b]	98.5 ± 6.3	98.2 ± 7.6	100.1 ± 6.4	100	107.1 ± 14.4	97.4 ± 7.0	101.5 ± 5.9	102.7 ± 10.5	100	103.4 ± 11.1	97.0 ± 7.6	95.0 ± 8.1	97.2 ± 10.9	100[a]	123.8 ± 25.6	104.0 ± 7.8	97.1 ± 6.9[c]	98.6 ± 10.8

Human whole blood (n = 10) was treated with Pg-3-glc, PCA, 4-HBA, PGA, or vehicle control at concentrations of 0-5 μmol/L, prior to LPS stimulation (1 μg/mL), and incubated for 24 hours at 37°C. Cytokine concentrations in the culture supernatants were measured using a cytometric bead array kit. Results are expressed as percentage of cytokine concentration vs control (no polyphenols). Data are represented as the means ± SD. Data were analyzed by 1-way ANOVA and Dunnett post hoc analysis, where applicable, and a lowered $P < .01$ was considered significant to account for multiple comparisons. Statistically significant differences are denoted as [a]$P < .01$ (1-way ANOVA), [b]$P < .01$ vs control (Dunnett post hoc analysis), and [c]$P < .001$ vs control (Dunnett post hoc analysis).

effects of any of the test compounds on oxidative burst activity upon *E coli* stimulation. In contrast, a reduction in hydrogen peroxide production was reported by a raspberry fruit extract in phorbol-12-myristate-13-acetate–stimulated J774 murine macrophages, but the effects were only observed at higher extract concentrations and were less pronounced in arachidonic acid–stimulated macrophages [41]. Similarly, diminished production of reactive oxygen species production was reported by a number of anthocyanin- and flavonoid-rich fruits by opsonized zymosan-activated phagocytes, but no effects were observed with phorbol-12-myristate-13-acetate as stimulus [13]. This study used higher polyphenol doses compared with the current experiment, where doses may have been too low to have an effect. The 2 studies have also used a different technique for reactive oxygen species measurement. Another important consideration that may contribute to discrepant findings between studies might be the nature of the stimulus used. Emerging data suggest that the phagocytic immune response is governed by the type of stimulus [42]. It is important to note that interpretation of oxidative burst data is not straightforward. On the one hand, oxidative burst is involved in the destruction of pathogens upon phagocytosis and thus represents a critical component of immune defense [4], but it can also be harmful to tissues and contribute to the pathogenesis of chronic health conditions [13], especially if there are insufficient antioxidant defenses.

Cytokines are a critical component of immune defense, but, on the other hand, inappropriate or excessive production of TNF-α, IL-1β, IL-6, and IL-8 has been linked with the pathogenesis of a number of chronic inflammatory diseases [4]. IL-10, on the other hand, is a predominantly anti-inflammatory cytokine and would be expected to be associated with reduced atherosclerosis by suppressing macrophage activation and inhibiting several proinflammatory cytokines, chemokines, and growth factors [43]. In the current experiment, the increased IL-10 production by Pg-3-glc and PGA could be interpreted as a modest anti-inflammatory effect, and the original hypothesis is therefore partially upheld [43]. Interestingly, in the current experiment, the increase in IL-10 by Pg-3-glc and PGA was only observed at the lowest dose tested (0.08 μmol/L). Importantly, these doses are physiologically relevant. PGA and Pg-3-glc were reported in plasma upon anthocyanin consumption at 5-600 nmol/L [29,30,33,34]. Although the validity and interpretation of this effect remain to be confirmed, it could indicate the presence of an inverted U-shaped response curve. Previously, trends for (inverted) U-shaped associations were observed in a cell model testing vanillic acid and heme oxygenase-1 protein expression [44] in a human intervention study between blueberry beverage consumption and flow-mediated dilation [45] and in an epidemiological study between tea consumption and coronary heart disease mortality [46]. To our knowledge, no other studies have investigated the effect of PGA on IL-10 production by monocytes or macrophages. There were no effects of any of the other test compounds on IL-10 levels, which are in line with results on the effect of PCA in human monocyte–derived dendritic cells [25] and PCA and 4-HBA in THP-1 monocytes [47]. In the latter study, Pg-3-glc did not alter IL-10 production, which is in contrast to the current data. However, that study only tested a 1-μmol/L dose,

and the lack of effect was consistent with the observation that Pg-3-glc at 0.31-5.00 μmol/L had no effect in the current study.

There is evidence from in vitro studies to suggest that particular structural characteristics might be required for phagocytosis-enhancing effects. A cell line study (using 1,25-dihydroxyvitamin D3–differentiated HL60 cells) concerned with the effect of green tea polyphenols suggested that a pyrogallol-type B-ring and/or a galloyl group is required to increase phagocytic activity [11]. These structural characteristics were absent from the tested compounds in the current studies. However, this observation was only based on a screen of 6 compounds and therefore requires confirmation, ideally in screening studies with a larger number of related compounds to clearly identify chemical structures or properties required for phagocytosis-enhancing effects.

A limitation of the present experiment is that although subjects were asked to follow a low-flavonoid diet for 24 hours prior to the blood sample collection, it cannot be excluded that some phenolic acids were present in circulation, which could have contributed to the variability and/or lack of effect.

In conclusion, there was no effect of the strawberry-derived anthocyanin Pg-3-glc or 3 of its physiologically relevant plasma metabolites on phagocytosis or oxidative burst activity in an in vitro human whole blood culture model. The data suggest that PGA and Pg-3-glc at physiologically attainable concentrations may possess anti-inflammatory properties through modulation of IL-10 production, which could contribute to protective effects in inflammatory diseases, although the magnitude of the effects appears to be modest and was only observed at the lowest dose tested (0.08 μmol/L). None of the test compounds had any effect on IL-1β, TNF-α, IL-6, and IL-8. Subsequent studies should explore other immunomodulatory effects of dietary anthocyanins.

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Appendix 2: Published abstract

Amini AM, Rodriguez-Mateos A, Spencer JPE, Yaqoob P. Stability of anthocyanins *in vitro*. *Proceedings of the Nutrition Society* 2013; 72 (OCE4): E198.

Appendix 3: Published book chapter

Corona G, Vazour D, **Amini A**, Spencer JPE. The impact of gastrointestinal modifications, blood-brain barrier transport, and intracellular metabolism on polyphenol bioavailability: an overview. In: Watson RR, Preedy VR, Zibadi S, ed. Polyphenols in human health and disease. London: Elsevier, 2013: 591-604.

Appendix 4: RCT ethics application

Application Form

SECTION 1: APPLICATION DETAILS

1.1

Project Title: Bioavailability of blackcurrant-derived anthocyanins and effects on cardiovascular function (Simplified title to be used in communications with potential volunteers: "Effect of blackcurrant juice on cardiovascular health" or "Blackcurrant juice study")

Date of Submission: 14/02/2014 Proposed start date: April 2015 Proposed End Date: December 2015

1.2

Principal Investigator: Professor Parveen Yaqoob, University of Reading

Office room number: 2-55 Internal telephone: 8720

Email address: p.yaqoob@reading.ac.uk Alternative contact telephone: 0118 378 8720
(Please note that an undergraduate or postgraduate student cannot be a named principal investigator for research ethics purposes. The supervisor must be declared as Principal Investigator)

Other applicants
Name: Anna Amini (PhD student, Study Researcher)
Institution/Department: Food and Nutritional Sciences, University of Reading
Email: a.m.amini@pgr.reading.ac.uk

1.3

Project Submission Declaration

I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I understand that it is a legal requirement that both staff and students undergo Criminal Records Checks when in a position of trust (i.e. when working with children or vulnerable adults).

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed…… (Principal Investigator) Date:……

…… (Student) Date:……

…… (Other named investigators) Date:……

…… (Other named investigators) Date:……

1.4

University Research Ethics Committee Applications

Projects expected to require review by the University Research Ethics Committee must be reviewed by a member of the School research ethics committee and the Head of School before submission.

Signed…… (Chair/Deputy Chair of School Committee) Date:……

Signed…… (Head of Department) Date:……

Signed…… (SCFP Ethics Administrator) Date:……

SECTION 2: PROJECT DETAILS

2.1

Lay summary

Please provide a summary of the project in non-specialist terms, which includes a description of the scientific background to the study (existing knowledge), the scientific questions the project will address and a justification of these. Please note that the description must be sufficient for the committee to take a reasonable view on the likely scientific rigour and value of the project.

Anthocyanins (ACNs) are abundant in berry fruits and they belong to the flavonoid group of phytochemicals. A cardio-protective benefit of flavonoid-rich foods has been demonstrated in several studies (Hooper et al., 2008). However, the most convincing clinical evidence exists only for a few flavonoid-rich products (chocolate, black and green tea), which have been extensively studied. Consumption of these flavonoid sources has been reported to exert beneficial effects on some risk factors for cardiovascular disease, such as LDL cholesterol, blood pressure (BP), and endothelial function. With regards to anthocyanins, there is observational evidence that has linked their consumption with a lower risk of acute coronary events and stroke (Cassidy et al., 2013), but only a few clinical trials have investigated the effect of anthocyanin-rich foods on cardiovascular disease risk (Rodriguez-Mateos 2013). Therefore the present study aims to to fill this knowledge gap by investigating the effect of blackcurrant-derived anthocyanins on cardiovascular function in a middle-aged population.

References

Cassidy A, Mukamal KJ, Liu L, Franz M, Eliassen AH, Rimm EB. High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. Circulation 2013;127:188-96.

Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, Ryder JJ, Hall WL, Cassidy A. Flavonoids, flavonoid-rich foods, and cardiovascular risk: A meta-analysis of randomized controlled trials. Am J Clin Nutr 2008;88:38-50.

Rodriguez-Mateos A, Heiss C, Borges G, Crozier A. Berry (Poly)phenols and Cardiovascular Health. J Agric Food Chem 2013; In Press.

2.2

Procedure

Please briefly describe what the study will involve for your participants and the procedures and methodology to be undertaken (*you may expand this box as required*).

An acute study is proposed to determine the anthocyanin bioavailability from a blackcurrant juice beverage and its acute effects on vascular function, platelet function and inflammation.

Endpoint measurements:

- Anthocyanins and their metabolites in blood samples
- Vascular function: Flow-mediated dilatation (FMD) and DVP
- Plasma total nitrate and nitrite
- Plasma cytokine levels
- Blood pressure
- Platelet aggregation
- Metabolomics on urine and plasma samples

Secondary Objective:
To correlate potential effects on cardiovascular markers / inflammation with circulating levels of juice-derived anthocyanins and their metabolites.

Study design
The proposed study will be a double-blind, placebo-controlled crossover intervention (see Figure 1), where volunteers will be required to attend the Hugh Sinclair Unit of Human Nutrition on two study days. Volunteers will be instructed to follow a low flavonoid diet for 24 h hours before and during each study day by omitting foods from a prescribed list and to fast overnight (for 12 hours). Volunteers will also be given and asked to consume a low fat evening meal provided by the University the evening before each study day. Volunteers will also be asked to refrain from exercise and alcohol 24 h prior to each study visit. On arrival at the Hugh Sinclair Unit of Human Nutrition, subjects weight and height will be measured and they will be asked what medications they have taken since their last visit. Subjects will then rest for 30 minutes in a quiet, temperature controlled room and during this time a 24 h dietary recall (Appendix J) will be taken to check compliance to a flavonoid free period. After this 30 minute period, a cannula is inserted in the volunteer's non-dominant arm. A baseline blood sample will be collected (for analysis of anthocyanins and their metabolites and for cytokine, nitric oxide and platelet function) and baseline vascular measurements will be performed (FMD, DVP and blood pressure). Subjects will also be asked for a spot urine sample. The subjects will then be given a low-flavonoid breakfast together with the intervention drink (200 ml containing 600 mg ACNs) or the placebo product. From this point onwards subjects will be asked to collect all urine passed for a period of 24 hours (urine will be collected on site at 1, 2, 4 and 6 hours after drink consumption and as combined collection during 6 and 24 h). Blood samples will be collected at, 1, 2, 4 and 6 hours after the consumption of the drink and the cannula removed. FMD vascular function and blood pressure will be measured at, 1, 2, 4 and 6 hours post ingestion of the treatment. DVP will be measured at 2, 4 and 6 hours post ingestion of the treatment. Platelet function will be measured at 2 and 4 hours after consumption of the drink. A standard lunch with low flavonoid content will be provided after the 2 h measurements. Volunteers will be asked to follow a low flavonoid diet until the morning after the study day and will be provided with a low-flavonoid meal to eat for dinner. Subjects will be asked to return to the unit for a final blood collection the next morning (24 h) and to deliver their urine that has been collected since leaving the study site the previous day. This process will then be repeated by the volunteers on their second visit, when 200 ml of the other juice (either control or blackcurrant juice drink) will be consumed.

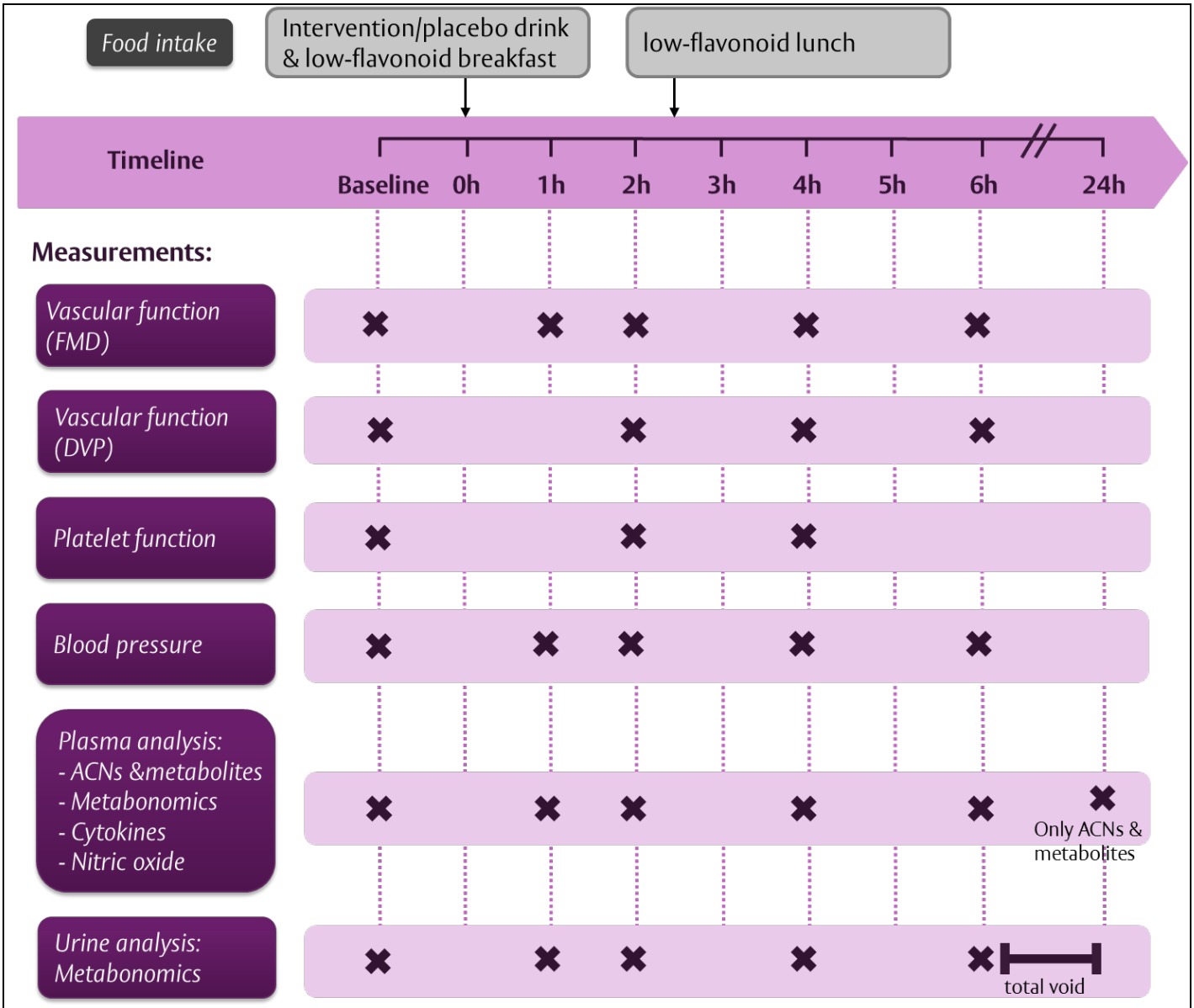

Figure 1. Study outline

Screening procedure

Interested volunteers will be asked to complete a health and lifestyle questionnaire (Appendix H) to assess their initial eligibility for the study. Participants who appear to meet the inclusion criteria will be provided with a participant information sheet (Appendix F) which outlines the details of the study. If the potential volunteer is still keen to participate, he/she will be asked to attend a screening session at the Hugh Sinclair Unit of Human Nutrition at the University of Reading. During this screening appointment all the procedure will be explained in detail. Participants will be encouraged to ask any question and if they are willing to proceed they will be asked to sign the Consent Form (Appendix G) which will be also signed by the researcher. During this screening session weight, height, blood pressure and waist-hip circumference will be measured. Then, the participant will be asked to provide a fasting blood sample (15 ml equivalent to 1 tablespoon) which will be taken by venepuncture from the antecubital vein by a nurse or experienced phlebotomist for the measurement of blood count, lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides), glucose levels and markers of liver & kidney function (creatine, bilirubin, uric acid, ALT, GGT and ALP). After the blood collection, participants will be encouraged to eat breakfast

in the unit. Following the screening session the potential volunteers will be contacted by one of the study investigators and informed whether they are eligible to take part.

Inclusion Criteria:
Subjects suitable for recruitment should be aged 30-55 years, non-smokers, have a BMI ranging from 20 to 30 and be generally healthy as established by a 'health and lifestyle' questionnaire and a screening blood sample.

Exclusion Criteria:

- Diabetes mellitus
- Heart problems, stroke, vascular disease
- Inflammatory disease
- Kidney, liver, pancreas or gastrointestinal diseases.
- Medication for hyperlipidaemia, hypertension, hypercoagulation, inflammatory conditions
- Asthma
- Allergies
- Smokers (social smokers who agree to abstain for 1 month before and during the study not excluded)
- Taking phytochemical, antioxidant or fish oil supplements (unless willing to stop for the study period)
- Taking aspirin > 2 times per month and unwilling to abstain from aspirin ingestion for 14 days prior each study visit
- History of alcohol misuse
- Consumption of alcohol >21 units (men) or >15 units (women
- Intense aerobic exercise >20 min 3 x per week
- Participation in another clinical trial
- Antibiotics in previous 3 months before study
- Low haemoglobin levels
- Females who are pregnant, lactating, or if of reproductive age and not using a reliable form of contraception (including abstinence).
- An employee of the sponsor or members of their immediate family.

2.3

Where will the project take place?

The study will be conducted at the Hugh Sinclair Unit of Human Nutrition, Department of Food and Nutritional Sciences, University of Reading where numerous human studies have been safely and successfully completed.

If the project is to take place in Hugh Sinclair Unit of Human Nutrition, please confirm that you have informed Ms Jan Luff (j.e.luff@reading.ac.uk).
I can confirm that Jan Luff has been informed (December 2013). Jan's successor Sarah Hargreaves has also been informed (January 2015).

2.4

Funding
Is the research supported by funding from a research council or other *external* sources (e.g. charities, business)? Yes

If Yes, please give details:
This study is supported by a BBSRC CASE studentship with GlaxoSmithKline as industrial sponsor.

Please note that *all* projects (except those considered as low risk, which would be the decision of the School's internal review committee and require Head of Department approval) require approval from the University Research Ethics Committee.

2.5

Ethical Issues
Could this research lead to any risk of harm or distress to the researcher, participant or immediate others? Please explain why this is necessary and how any risk will be managed.

The intervention and placebo products to be used in this study are made from normal components of our diet that are commercially available and thus, it is not expected that the volunteers will experience any adverse effects. Moreover, all the procedures of the study (venepuncture, FMD measurements) will be performed by trained researchers and in accordance of the relevant SOPs which also indicate the necessary actions in the case of an emergency (e.g. fainting).

2.7

Payment
Will you be paying your participants for their involvement in the study? Yes~~/No~~ (delete)
If yes, please specify and justify the amount paid

Each participant will receive an honorarium of £100 in order to cover time and travel expenses. There is no payment for the initial screening visit. Early dropouts will be paid on a pro-rata basis.

Note: excessive payment may be considered coercive and therefore unethical. Travel expenses need not to be declared.

2.8

Data protection and confidentiality
What steps will be taken to ensure participant confidentiality? How will the data be stored?

A coding system will be used for all participants and their samples. All personal data will be stored safely in a dedicated computer using a specific password, known only by the principal investigator and the study researcher. Moreover, the names of the volunteers will not be saved on the same file with the rest of the data. Finally, all the completed consent forms will be saved in a safe locker.
The Department of Food and Nutritional Sciences is fully licensed under the Human Tissue Act 2004 and as such will adhere to the guidelines necessary for the storage of urine.

2.9

Consent

Please describe the process by which participants will be informed about the nature of the study and the process by which you will obtain consent

Interested volunteers will be asked to complete a health and lifestyle questionnaire (Appendix H). Participants who are suitable for inclusion in the screening phase of the study will be provided with a participant information sheet which outlines the details of the study. If the potential volunteer is still keen to participate, he/she will be asked to attend a screening session at the Hugh Sinclair Unit of Human Nutrition at the University of Reading. During this screening appointment all the procedure will be explained in detail. Participants will be encouraged to ask any question and if they are willing to proceed they will be asked to sign the Consent form (Appendix G) which will be also signed by the researcher. A copy of these forms will be kept by the subject and in a secure place at The University of Reading (for a period of 5 years).

2.10

Genotyping

Are you intending to genotype the participants? Which genotypes will be determined?

No.

Please note that a copy of all information sheets on the implications of determining the specific genotype(s) to be undertaken must be appended to this application.

SECTION 3: PARTICIPANT DETAILS

3.1

Sample Size

How many participants do you plan to recruit? Please provide a suitable power calculation demonstrating how the sample size has been arrived at or a suitable justification explaining why this is not possible/appropriate for the study.

The sample size calculation was performed for the two main endpoint, vascular reactivity measured by FMD and platelet function. A total of 21 subjects will be required and it is planned to recruit 25 volunteers to allow for dropouts.

15 subjects is a sufficient sample size to detect a 0.7% change in FMD measurements. This calculation is based on the assumption that the within standard deviation is 0.6 (according to previous studies in the department) and a statistically significant difference of 0.7% would be of interest. The significance level alpha was chosen at 0.05 and the power was set at 80%.

21 subjects is a sufficient sample size to detect a 6% change in agonist-induced platelet aggregation. This calculation is based on the assumption that the standard deviation is 5.5% (Freedman et al. (2001) observed standard deviations between 3.3% and 7.4%) and a statistically significant difference of 6% would be of

interest. Previous studies concerned with the effect of flavonoids on platelet aggregation observed 3 and 15% reductions in agonist-induced platelet aggregation (Erlund 2008, Ostertag 2013). The significance level alpha was chosen at 0.05 and the power was set at 80%.

Reference

Erlund I, Koli R, Alfthan G, Marniemi J, Puukka P, Mustonen P, Mattila P, Jula A. Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. AJCN 2008; 87: 323–331.

Freedman JE, Parker C, Li L, Perlman JA, Frei B, Ivanov V, Deak LR, Iafrati MD, Folts JD. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circ 2001; 103: 2792-2798.

Ostertag LM, Kroon P, Wood S, Horgan GW, Cienfuegos-Jovellanos E, Saha S, Duthie GG, de Roos B. Flavan-3-ol-enriched dark chocolate and white chocolate improve acute measures of platelet function in a gender-specific way - a randomized-controlled human intervention trial. Mol Nutr Food Res 2013; 57: 191–202.

Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, Kelm M. (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. PNAS 2006; 103: 1024-1029.

3.2

Will the research involve children or vulnerable adults (e.g. adults with mental health problems or neurological conditions)? ~~Yes/~~No ~~(delete)~~

If yes, how will you ensure these participants fully understand the study and the nature of their involvement in it and freely consent to participate?

(Please append letters and, if relevant, consent forms, for parents, guardians or carers). Please note: information letters must be supplied for all participants wherever possible, including children. Written consent should be obtained from children wherever possible in addition to that required from parents.

3.3

Will your research involve children under the age of 18 years? ~~Yes/~~No ~~(delete)~~
Will your research involve children under the age of 5 years? ~~Yes/~~No ~~(delete)~~

3.4

Will your research involve NHS patients, Clients of Social Services or will GP or NHS databases be used for recruitment purposes? ~~Yes/~~No ~~(delete)~~

Please note that if your research involves NHS patients or Clients of Social Services your application will have to be reviewed by the University Research Ethics Committee and by an NHS research ethics committee.

3.5

Recruitment

Please describe the recruitment process and append all advertising and letters of recruitment.

- Use of our department's volunteer database containing approximately 2000 volunteers who have indicated that they would be interested in participating in future trials (Appendix A).
- Email advertisement to University of Reading staff and students and to staff members in large local organisations and companies such as Reading Borough Council, The Prudential, Oracle and ING Direct (Appendix B).
- Posters and leaflets in public places, such as around the university campus or in community centres (Appendix D, Appendix E).
- Advertisement in local newspapers, magazines and websites (Appendix C).
- Letter advertisement to potential volunteers on sensory dimension's database (Appendix B).

Important Notes

1. The Principal Investigator must complete the Checklist in Appendix A to ensure that all the relevant steps and have been taken and all the appropriate documentation has been appended.

2. If you expect that your application will need to be reviewed by the University Research Ethics Committee you must also complete the Form in Appendix B.

3. For template consent forms, please see Appendices C.

4. For examples of information letters, see Appendices D

Appendix A: Application checklist

This must be completed by an academic staff member (e.g. supervisor)

Please tick to confirm that the following information has been included and is correct.
Indicate (N/A) if not applicable:

Information Sheet

Is on headed notepaper	☑	
Includes Investigator's name and email / telephone number	☑	
Includes Supervisor's name and email / telephone number	☑	
Statement that participation is voluntary	☑	
Statement that participants are free to withdraw their co-operation	☑	
Reference to the ethical process	☑	
Reference to Disclosure	☐	N/A ☑
Reference to confidentiality, storage and disposal of personal information collected	☑	
Consent form(s)	☑	

Other relevant material

Questionnaires	☑	N/A ☐
Advertisement/leaflets	☑	N/A ☐
Letters	☑	N/A ☐
Other (please specify): Study dietary modifications	☑	N/A ☐

Expected duration of the project (months) 12

Name (print) Professor Parveen Yaqoob…… Signature ……

University Research Ethics Committee

Appendix B

Project Submission Form

Note All sections of this form should be completed. Please continue on separate sheets if necessary.

Principal Investigator: Professor Parveen Yaqoob

School: School of Chemistry, Food and Pharmacy

Title of Project: Bioavailability of blackcurrant-derived anthocyanins and effects on cardiovascular function. (Simplified title to be used in communications with potential volunteers: "Effect of blackcurrant juice on cardiovascular health" or "Blackcurrant juice study")

Proposed starting date: April 2015

Brief description of Project:

A randomised, placebo-controlled, crossover intervention study examining the bioavailability of anthocyanins from a blackcurrant juice beverage and acute effects on cardiovascular function in a middle-aged population.

I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed…….(Investigator) Date……

…….(Head of Department) Date……

…….(Student) Date……
(Where applicable)

Checklist

1. This form is signed by my Head of School ☑

2. The Consent form includes a statement to the effect that the project has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee, and has been allowed to proceed ☑

3. I have made, and explained within this application, arrangements for any confidential material generated by the research to be stored securely within the University and, where appropriate, subsequently disposed of securely. ☑

4. I have made arrangements for expenses to be paid to participants in the research, if any, OR, if not, I have explained why not. ☑

5. EITHER

   (a) The proposed research does not involve the taking of blood samples; ☐

   OR

   (b) For anyone whose proximity to the blood samples brings a risk of Hepatitis B, documentary evidence of protection prior to the risk of exposure will be retained by the Head of School. ☑

   Signed…….(Head of Department) Date……

6. EITHER

   (a) The proposed research does not involve the storage of human tissue, as defined by the Human Tissue Act 2004; ☐

   OR

   (b) I have explained within the application how the requirements of the Human Tissue Act 2004 will be met. ☑

7. EITHER

   (a) The proposed research will not generate any information about the health of participants; ☐

OR

(b) In the circumstance that any test reveals an abnormal result, I will inform the participant and, with the participant's consent, also inform their GP, providing a copy of those results to each; ☑

OR

(c) I have explained within the application why (b) above is not appropriate. ☐

8. EITHER

(a) the proposed research does not involve children under the age of 5; ☑

OR

(b) My Head of School has given details of the proposed research to the University's insurance officer, and the research will not proceed until I have confirmation that insurance cover is in place. ☐

Signed…….(Head of Department) Date……

This form and further relevant information (see Sections 5 (b)-(e) of the Notes for Guidance) should be returned to, Ms. Tasha Dawson, Senior Academic Services Administrator, Room 212b, Whiteknights House. You will be notified of the Committee's decision as quickly as possible, and you should not proceed with the project until then.

The following Appendices are included in the Application Form

Appendix A: Email-contact to potential volunteers from the Hugh Sinclair database

Appendix B: Advertising Letter/email to potential volunteers other than those on the Hugh Sinclair database

Appendix C Advertisement in local newspapers, websites and/or magazines

Appendix D: Poster advertisement

Appendix E: Leaflet advertisement

Appendix F: Participant information sheet

Appendix G: Consent Form

Appendix H: Health and Lifestyle questionnaire

Appendix I: Study dietary modifications

Appendix J: 24 h dietary recall

Appendix A: Email-contact to potential volunteers from the Hugh Sinclair database

Re: Blackcurrant juice study

Dear ***xxx***

When you took part in a previous human nutrition research study at the Hugh Sinclair Unit of Human Nutrition (University of Reading), you expressed an interest in hearing about new studies taking place, for which you might be eligible. We are currently recruiting for a new nutrition study:

The effect of blackcurrant juice on cardiovascular health

In order to take part you must be:

- Male or female aged 30-55 years
- non-smoker
- in good general health
- free from any significant current or previous medical history

The study will take place from April and involve three visits: one screening visit and two whole day study visits.

You will be remunerated for your time and travel expenses.

If you are interested in taking part in this study, please contact Sarah Hargreaves on 0118 378 7771 or Anna Amini on a.amini@reading.ac.uk

Yours sincerely,
Anna Amini

Appendix B: Advertising Letter/email to potential volunteers other than those on the Hugh Sinclair database

Re: Blackcurrant juice study

Dear ***xxx***

We are seeking volunteers willing to take part in a human nutrition study:

The effect of blackcurrant juice on cardiovascular health

In order to take part you must be:

- Male or female aged 30-55 years
- non-smoker
- in good general health
- free from any significant current or previous medical history

The study will take place from April and involve three visits: one short screening visit and two whole day study visits.

You will be remunerated for your time and travel expenses.

If you are interested in taking part in this study, please contact Sarah Hargreaves on 0118 378 7771 or Anna Amini on a.amini@reading.ac.uk

Yours sincerely,
Anna Amini

Appendix C: Advertisement in local newspapers, websites and/or magazines

The University of Reading is recruiting healthy men and women, aged 30-55, for a human nutrition study investigating the effect of compounds from berries on heart health. Volunteers should be willing to attend a short screening visit and two whole day study visits.
For more information please contact *contact name* at *email* or *tel.*

Appendix D: Poster advertisement

Appendix E: Leaflet advertisement

Appendix F: Participant information sheet

Participant information sheet

Blackcurrant juice study

You have been invited to take part in a study investigating the effect of compounds from berries on cardiovascular health (Blackcurrant juice study). Before you decide to participate please take time to read the following information carefully. Please ask us if there is anything that is not clear or if you would like more information.

Contact Name: Anna Amini (PhD student, Study Researcher)
Food and Nutritional Sciences Department,
School of Chemistry, Food and Pharmacy
The University of Reading, PO Box 226, Whiteknights
Reading RG6 6AP United Kingdom

Room 2-1

Tel: *add study mobile number*
Email: a.amini@reading.ac.uk

What is the purpose of the study?

Many studies have shown that a regular consumption of fruits and vegetables may improve human health and reduce the risk of chronic diseases such as, heart diseases, certain cancers and type 2 diabetes. We are particularly interested in the health benefits of berry fruits and our study aims to establish the effect of a blackcurrant drink on cardiovascular health (health of blood vessels and inflammation).

Am I suitable to take part?

We are aiming to recruit male and female subjects who are generally fit and healthy. Subjects suitable for recruitment to the study should be aged 30-55 years, non-smokers, have a Body Mass Index (BMI) of 20-30, have a normal liver and kidney function and haematology and a max weekly alcohol intake of <15 units (women) / <21 units (men). A unit of alcohol is half a pint of beer/lager, a single pub measure of spirits e.g. gin/vodka or a small glass of wine (125 ml).

You will not be able to take part if you:

- Have diabetes mellitus.
- Have heart problems.
- Have inflammatory disease.
- Have kidney, liver, pancreas or gastrointestinal disease.
- Have medical history of cardiovascular disease, including coronary heart disease (angina and heart attack) and stroke.
- Have asthma.
- Have allergies.
- Take drug treatment for hyperlipidaemia, hypertension, inflammation or hypercoagulation.
- Smoke

- Take aspirin > 2 times per month and are unwilling to abstain from aspirin ingestion for 14 days prior each study visit
- Take any phytochemicals, antioxidants, fish oil supplements.
- Have a history of alcohol misuse.
- Participate in intense aerobic exercise.
- Participation in another clinical trial within the last three months.
- Took antibiotics for the previous 3 months.
- Are pregnant, lactating, or if of reproductive age and not using a reliable form of contraception (including abstinence).

Do I have to take part?
It is up to you to decide whether to take part or not. If you decide to take part you will be given this information sheet to keep and asked to sign a consent form. Your participation remains purely voluntary, you may withdraw at any stage and without giving a reason.

What would happen to me if I take part?
Before being admitted onto the study, your initial eligibility will be determined by completion of a health & lifestyle questionnaire over the telephone. Alternatively, it can be filled in online if it is more convenient. If you appear to meet the criteria for this study you will be provided with a participant information sheet which outlines the details of the study. If you are still keen to participate in the study, you will be asked to attend a screening session at the Hugh Sinclair Unit of Human Nutrition at the University of Reading.

Upon arrival at the Hugh Sinclair Unit of Human Nutrition, you will be provided with further information about the study and we will answer any questions that you might have. You will be asked to complete a consent form and will have your height, weight, body composition and blood pressure measured. We will also collect a small blood sample (~15 ml, one tablespoon). Following this screening session you will be contacted by one of the study investigators and informed whether you are eligible to take part.

If you agree to take part in this study and are eligible, you will be asked to visit the Hugh Sinclair Unit of Human Nutrition at the University of Reading to take part in the study on <u>two separate occasions, each lasting approximately 10 hours</u>.

Before each study visit

- You will also be asked to abstain from aspirin ingestion during the 14 days before and during the study day.
- You will also be asked to abstain from alcohol and certain foods (a list will be provided) during the 24 hours before and during the study day.
- You will be asked to minimise changes to your usual lifestyle and avoid strenuous exercise during the 24 hours prior to the study day.
- You will need to follow the above guidelines until you have provided the last urine sample (24 hours after starting the study day).
- You will be asked to consume a standardised meal the evening before the study visit (finish consumption at least 12h prior to study visit). A selection of commercial dinners will be provided. Please let the investigators know if you have food allergies or specialised dietary requirements so these can be managed.

- You will be requested not to eat or drink anything other than water 12 hours before the study visit until you arrive at the Hugh Sinclair Unit of Human Nutrition the next morning.
- You will be provided the water to consume 12 hours before the study visit
- Water consumption is not restricted

During each study visit

- You will be given an appointment for 08.00am or 09.30am to arrive at the Hugh Sinclair Unit of Human Nutrition. You will be asked to arrive fasted (consume no food or beverage with the exception of water for the previous 12 hours).
- Your weight and body composition will be measured.
- You will then rest for 30 minutes in a quiet temperature-controlled room to ensure that you are calm and relaxed. During this time, we will ask you what you have eaten during the last 24 h to make sure you comply to the dietary requirements.
- A small flexible plastic cannula (a small tube which sits inside a vein in the forearm) will be inserted by an experienced, trained phlebotomist, and will remain in your arm with minimal discomfort to allow us to take blood samples during the study day. However, if the cannula becomes uncomfortable before the end of the study day it will be removed and, only if it will not cause you distress, blood samples will be collected by individual venepuncture instead.
- An initial blood sample will be drawn from the cannulated vein shortly before breakfast.
- Your blood pressure and blood vessel function will be measured in the non-cannulated arm using two non-invasive, safe and pain-free techniques called FMD and DVP.
- You will then be fed a breakfast together with the test beverage.
- Throughout the test day, blood samples will be taken from the cannula at 1h, 2h, 4h and 6h after the test beverage consumption. The total volume collected over the study day will be 195 ml (corresponding to 13 tablespoons). You will also be asked to give a single blood sample of around 12 ml (approximately 1 tablespoon) the next morning after your visit, which will be taked by venepuncture.
- You will be asked to collect your urine at different time points during the study day (0h, 1h, 2h, 4h and 6h after consumption of the test drink). You will be given a urine container in a cool bag to take home when you finish your visit to collect all urine produced till the following morning when you come to the Hugh Sinclair Unit of Human Nutrition to give the last blood and urine sample.
- At 1h, 2h, 4h and 6h after the test beverage consumption your blood vessel function will be measured using the FMD machine and at 2h, 4h and 6h using the DVP machine.
- After you have completed the 2h measurements you will be given a standard lunch.
- After the last blood sample is taken, the phlebotomist will remove the cannula from your arm.
- You will be asked to follow the dietary restrictions until the morning after the study day and will be provided with a meal to eat for dinner.
- You will be asked to return to the unit for a final blood collection the next morning (24 h) and to deliver the urine that has been collected since leaving the study site the previous day.

What will be measured in the blood and urine samples collected?

- The blood sample collected at the screening visit will be used to measure levels of blood fats, glucose, markers of kidney and liver function and to perform a full blood count. This is necessary to further assess your suitability for participation in the study.

- The blood samples collected during the study visits will be used to measure the concentration of specific blackcurrant compounds and their metabolites. They will also be used for the measurement of markers of heart disease risk.

How will my vascular function be measured?

You will be required to lie horizontally on the bed before the measurements begins. The first technique, called flow-mediated dilatation (FMD , uses an ultrasound system to visualise the artery in the upper arm from which its diameter can be measured. A cuff similar to those used to measure blood pressure will be placed just below your elbow and inflated until the pressure or tightness of the blood pressure cuff prevents the blood from entering your forearm. It will be held at this pressure for 5 minutes. When the pressure is released, the artery widens. The wider the artery after the release of pressure compared to before the pressure is applied implies a healthier artery. The high pressure of the blood pressure cuff is a little uncomfortable and you may experience 'pins and needles' or slight numbness in your fingertips towards the end of the 5 minutes. The level of discomfort will be similar to that associated with having your blood pressure taken. The procedure will take approximately 30 minutes per measurement.

The second type of measurement is the 'Digital Volume Pulse (DVP)'. The DVP is recorded by placing a clip on your index finger which uses infra-red light to measures the changes in the volume of blood in the fingertip. This painless measurement will be recorded 3 times over 10 second periods with 3 minute intervals between measurements.

What are the possible disadvantages of taking part?

Blood sampling is an invasive procedure, so there can be a small discomfort as any blood sampling which affects some people more than others. You should not experience any pain during or after this procedure. You may develop a small bruise at the site of the blood sample, but this will fade like any bruise. This procedure will be performed by a fully trained researcher or nurse in accordance with the University of Reading guidance on research involving the taking of blood samples.

The FMD and DVP techniques used for measuring vascular function are non-invasive and safe. However, there may be a small amount of discomfort during the FMD measurement during the inflation of the blood pressure cuff around your arm, but this is applied for no longer than 5 minutes at any one time and does not cause any damage to your arm or blood vessels.

What are the possible benefits of taking part?

You will be participating in research which will contribute to the body of knowledge on physiological responses to food intake.

Would my taking part in this study be kept confidential?

Confidential information will be stored securely and can only be accessed by the study investigators. All information collected during the study will be treated in strict confidence in accordance with the relevant data protection legislation. No information will be disclosed in any way that will allow identification of yourself.

Collected data will be shared with GlaxoSmithKline in a form which will not allow identification of the study participants. Anonymised research data will be available to the scientific community for subsequent research. However, there may be circumstances where delays on data sharing will be required, for example to safeguard intellectual property and to protect opportunities for

commercialisation. Third parties from GlaxoSmithKline or appropriate regulatory authorities may be granted direct access to the participants' data; however this will be carried out without violating the confidentiality of the participants.

Involvement of your General Practitioner (GP)
With your consent, your participation in the study and screening results will be forwarded to your GP. This will include information such as blood fat levels and blood pressure.

Will the results be available to me?
All your screening results will be available to you on request. Once the study is complete and analysed statistically we can provide you with some feedback about what we have found in the study and what it may mean for future research.

Do I have to modify my diet or lifestyle in any way?
The day prior to the study until the morning after the study day (last collection of blood and urines) you will be asked to avoid consuming certain foods (list will be provided). You will be also asked to refrain from alcohol, and consume a low fat evening meal as indicated above. These diet changes will have no effect on your general health and will be fully explained to you by the investigator. You can do your everyday activities. We do ask you to inform us if during the course of the study you are ill, and/or prescribed any medication.

What would happen to the results of the research?
The results of this study will contribute to the PhD thesis of a postgraduate student and will be published anonymously in scientific journals, oral presentations or other scientific contributions.

The results of this study will also be provided to GlaxoSmithKline, who are part-funding this study.

Who is organising and funding the research?
This research is being organised by The University of Reading's Hugh Sinclair Unit of Human Nutrition, and funded by the Government's BBSRC (Biology and Biotechnology Scientific Research Council) and GlaxoSmithKline.

Will I get paid for taking part?
An honorarium will be paid as an inconvenience allowance of £100 upon completion of the study, which includes any travel costs you may incur. Participation in this study is entirely voluntary and you may withdraw from the study at any time and need not give any reason for doing so. We will ask you to sign a consent form to take part in this study.

Who reviewed this study?
This project has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

Who do I contact for further information or complaints?

Main point of contact:	*For formal complaints:*
Mrs Anna Amini (PhD student, Study Researcher)	**Professor Parveen Yaqoob (Principal Investigator)** Email: p.yaqoob@reading.ac.uk

Email: a.amini@reading.ac.uk Tel: *add study phone number* Address: Department of Food and Nutritional Sciences, PO Box 266, University of Reading, Whiteknights Campus, Reading, RG6 6AP Office: 2-01, Food Biosciences building	Tel: 0118 378 8720 Address: Department of Food and Nutritional Sciences, PO Box 266, University of Reading, Whiteknights Campus, Reading, RG6 6AP Office: 2-55, Food Biosciences building

Thank you for reading this information sheet. If you decide to take part in this study you will be given a copy of the information sheet and a signed consent form to keep.

Appendix G: Consent Form

Please sign with your initials in the boxes

1. I have read and had explained to me by …… the accompanying Information Sheet relating to the project **Effect of blackcurrant juice on cardiovascular health (Blackcurrant Juice Study)**. ☐

2. I have had explained to me the purposes of the project and what will be required of me, and any questions I have had have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation. ☐

3. I understand that participation is entirely voluntary and that I have the right to withdraw from the project any time, and that this will be without detriment to any care or services I may be receiving or may receive in the future. ☐

4. Only if you request us to do so, your participation in the study and screening results will be forwarded to your GP. In the circumstance that any test reveals an abnormal result, you will be informed and, with your consent, we will also inform your GP, providing a copy of those results. ☐

5. This project has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee, and has been given a favourable ethical opinion for conduct. ☐

6. I consent to an initial blood sample being taken for screening purposes, followed by a series of blood samples throughout the study at the times indicated on the accompanying information sheet. ☐

7. I have received a copy of this Consent Form and of the accompanying Information Sheet. ☐

8. I have had explained to me that consent for my contact details and personal information to be added to the Hugh Sinclair Unit of Human Nutrition Volunteer Database is entirely voluntary. Accordingly I consent as indicated below: ☐

   I consent to my contact details being stored on the Hugh Sinclair Unit of Human Nutrition Volunteer Database

   Yes ☐ No ☐

   I consent to my screening information (date of birth, height, weight, blood pressure smoking status, long-term use of medication, and blood test results such as levels of cholesterol, liver enzymes, haemoglobin, blood cell count) being stored on the Hugh Sinclair Unit of Human Nutrition Volunteer Database.

   Yes ☐ No ☐

Name ……..

Date of birth ……

Signed ……

Date ……

Witnessed by

Name……. Signature…….

Date……

GP details

Name……. Telephone: …….

Address:……

Appendix H: Health and Lifestyle questionnaire

Health and Lifestyle Questionnaire

Blackcurrant juice study

All information provided will remain confidential at all times.

Personal details

Name:		**Title**:
Address:		
Daytime Tel: **Evening Tel**: **Mobile no.**: **Best time to call**:	**Sex**: **Weight (kg)**: **Height (m)**:	**Age**: **DOB**:
E-mail:		

Medical or lifestyle question

Medical or lifestyle question	Circle as appropriate	Further details:
Have you recently received any form of vaccination? *If YES **postpone** participation into the study until 3 months have passed.*	YES NO	
Have you taken antibiotics within the last 3 months? *If YES **postpone** participation into the study until 3 months have passed.*	YES NO	
Do you suffer from allergies to any food, fruits and pollen? ***Exclude** if yes.*	YES NO	
Do you have a history of drug or alcohol misuse? ***Exclude** if yes.*	YES NO	
Have you taken part in any drug or nutritional research study in the last three months? *If YES **postpone** participation into the study until 3 months have passed.*	YES NO	must be >3 months
Do you smoke? If yes, would you be willing to completely abstain from smoking for the duration of the study? *Note: a cigarette-free period of 1 months is required (before starting the study)*	YES NO	

Exclude *if YES and not willing to abstain.*		
How much exercise do you participate in per week (not including walking)? *Please specify: i) type of exercise (this includes cycling to work), ii) how often & iii) duration* ***Exclude*** *if intense aerobic exercise >20 min 3 x per week.*	YES NO	
If female, are you pregnant, intending to become pregnant or breast-feeding? ***Exclude*** *if YES.*	YES NO	
If female, are you using contraception? *If yes, please give details.* ***Exclude*** *if not using adequate contraception. Adequate contraception is defined as abstinence, oral contraceptive, either combined or progestogen alone OR injectable progestogen OR implants of levonorgestrel OR estrogenic vaginal ring OR percutaneous contraceptive patches OR intrauterine device or intrauterine system OR double barrier method (condom or occlusive cap [diaphragm or cervical vault caps] plus spermicidal agent [foam, gel, film, cream, suppository]) OR male partner sterilization prior to the female subject's entry into the study, and this male is the sole partner for that subject.*		

Please tell us if you have any pre-existing health problems, including any of the following:

	Circle as appropriate	If yes, please give details:
Diabetes? ***Exclude*** *if YES*	YES NO	
Inflammatory, auto-immune disease or connective tissue disease? e.g. rheumatoid arthritis, lupus, psoriasis ***Exclude*** *if YES*	YES NO	
Do you have asplenia or any other acquired or congenial immunodeficiency? ***Exclude*** *if YES*		
Renal or bowel disease or history of liver disease or pancreatitis? ***Exclude*** *if YES*	YES NO	
Cirrhosis of the liver? ***Exclude*** *if YES*	YES NO	
Thyroid disorder? ***Exclude*** *if YES*	YES NO	
High blood pressure? ***Exclude*** *if YES*	YES NO	
High blood cholesterol? ***Exclude*** *if YES*	YES NO	
Heart problems, stroke or any vascular disease?	YES NO	

***Exclude** if YES*		
Any dermatological problems? ***Exclude** if disease might affect any study outcome measure.*	YES NO	
Any respiratory problems? e.g. asthma ***Exclude** if asthma or if disease might affect any study outcome measure.*	YES NO	
Any neurological conditions? ***Exclude** if disease might affect any study outcome measure.*	YES NO	
HIV? ***Exclude** if YES.*	YES NO	
Any form of cancer? *Accept if >5 yrs ago and no evidence of recurrence, or skin cancer operated in the past without major sequel (other than melanoma.*	YES NO	
Do you have any other health problems? ***Exclude** if disease might affect any study outcome measure.*	YES NO	
Do you take any prescription medication? ***Exclude** if on medication for hyperlipidaemia, hypertension, hypercoagulation and inflammatory conditions.*	YES NO	
Do you currently use any medication which affects your immune system e.g. oral prenisone or inhaled steroids? ***Exclude** if YES.*		
Do you take any pain killing medication (e.g. aspirin, paracetamol, nurofen, ibuprofen etc) daily or more than 4 times per week? ***Exclude** if use of any of above is prescribed or taken more than 4 times per week.*	YES NO	
Do you take aspirin more than 2 times per month? Are you willing to abstain from aspirin ingestion for 14 days prior to each study visit? ***Exclude** if taken more than 2 times per week and if unwilling to abstain from ingestion for 14 days prior to study visit.*	YES NO YES NO	

Dietary questions

	Circle as appropriate	Further details:
Are you a vegan or vegetarian? ***Exclude** vegans.*	YES NO	
Do you take any form of dietary supplements? For example, vitamins, minerals, antioxidants, phytochemicals or fish oil supplements. *If yes, please give details.* *Exclude if taking antioxidant, phytochemical or fish oil*	YES NO	

supplements and not willing to stop		
If you were to participate in our study, would you be willing to stop taking the supplements before and during the months of the study? *Note: before starting the study, a supplement-free period of 4 weeks is required.* ***Exclude** if NO.*	YES NO	
Do you drink alcohol? If yes, how many units do you roughly drink per week? *A unit of alcohol is half a pint of beer/lager, a single pub measure of spirits e.g. gin/vodka or a small glass of wine (125 ml).* ***Exclude** if weekly consumption of alcohol >21 units (men) or >15 units (women)*	YES NO	

Thank you for completing this questionnaire.

Appendix I: Study Dietary Modifications

Study dietary modifications

Blackcurrant Juice Study

Please EXCLUDE these foods from your diet 24 hours prior to each study visit, during the study day and until the morning after the study day (last blood and urine sample collection).	**Examples of foods you may INCLUDE in your diet.**
Fruit and vegetables: • Most fruits, in particular: berry fruits, orange, grapefruit, apple, pear, grapes, peach, apricot, nectarine, plum, exotic fruit. • Most vegetables, in particular: onion, peppers, tomato, broccoli, aubergine, beetroot, green beans, carrots • Olives • Jams and preserves **Beverages:** • All kinds of fruit juices (except study drink) • Tea (fruit, black, green, earl grey etc) • Coffee • All high energy and/or caffeinated drinks, eg: Coca-Cola, Red Bull, Lucozade • Alcohol • Red wine **Other:** • Soya products • Chocolate/cocoa • Dietary supplement e.g. fish oils, vitamins or minerals	**Fruit and vegetables:** • Potatoes • Sweetcorn • Mushrooms • Carrots • Bananas • Cucumber **Other:** • Rice • Pasta • Meat/fish • Dairy products: - Butter - Cheese - Milk - Yoghurt • White bread • Eggs

In addition, please do not ingest Aspirin for 14 days prior to the study visit!

Appendix J: 24 h dietary recall

24 h Dietary Recall

Blackcurrant Juice Study

Subject ID: **Subject initials:**

Visit: **Date:**

This is a recall of food and drink consumed in the last 24 hours.

Give Date and day (yesterday):

1. What was the first thing you had to eat or drink when you got out of bed yesterday?

2. What did you have to eat or drink at mid-morning?

3. What did you have to eat or drink during lunchtime?

4. What did you have to eat or drink in the afternoon between lunchtime and your evening meal?

5. What did you have to eat and drink for your evening meal?

6. Did you have anything to eat or drink after your dinner or before you went to bed?

7. Are there any snacks you had during the day you forgot to mention?

8. Have you ingested Aspirin in the last 14 days?

Researcher: _____ Date: _____