Interactions between anthocyanins, cholesterol metabolism and PON-1 in relation to HDL quality and CVD risk.

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Abstract

Background: Data from epidemiological studies demonstrate an inverse association between high intakes of anthocyanins and risk of cardiovascular disease. The human and animal dietary intervention studies also showed that consumption of anthocyanin-rich extracts and foods causes beneficial changes in lipid profiles. However, the potential mechanisms of action are yet to be fully elucidated; in particular, is it the anthocyanins per se or their metabolites that cause the changes, and what are the gene/protein targets in the lipoprotein metabolic pathways? Therefore, the aim of this research was to develop a mechanistic understanding of how anthocyanins and/or their metabolites alter lipid/lipoprotein metabolism and improve HDL function.

Approaches: Using cultured macrophages, the effects of the two main dietary anthocyanins and a large number of their metabolites on the expression of key genes involved in reverse cholesterol transport (RCT), and on the expression and enzyme activities of paraoxonase-1 (PON1) (marker of HDL function) were tested at physiologically relevant concentrations. The effects of anthocyanin consumption on cholesterol efflux transporters was investigated by quantifying the expression of ABCA1 and ABCG1 in aortic and liver tissues from ApoE$^{-/-}$ mice that had consumed diets supplemented with anthocyanin-expressing tomatoes compared to mice that had consumed diets supplemented with anthocyanin-free (control) red tomatoes. The broader effects of three major anthocyanin metabolites was assessed using RNA seq to study genome-wide changes in gene expression in cultured human macrophages. A randomized placebo-controlled crossover human intervention study was conducted using purified anthocyanin extracts to investigate the effects of consuming anthocyanins on lipid/lipoprotein profiles and biomarkers of HDL function and interactions with PON1 genotype.

Results: None of the tested anthocyanins or their metabolites significantly altered the expression of cholesterol transporter (ABCA1, ABCG1) or scavenger receptor (MSR1, SCARB1 and CD36) genes (the key genes involved in RCT), nor did they affect the expression of the PON1 gene or the activities of the enzyme. In addition, RNA seq analysis revealed that treatment with the three major anthocyanin metabolites did not cause any significant changes in the expression of macrophage genes or affect pathways related with atherosclerosis. In the human dietary intervention with high doses of isolated anthocyanins, no significant changes in lipid profiles, ApoA1, ApoB1, HDL subfractions and biomarkers of glycemic control were observed compared to the placebo after 4 weeks of intervention. The human

intervention also showed no significant interaction between the consumption of anthocyanin, biomarkers of HDL function, PON1 activities and PON1 genotype.

Conclusion: In conclusion, the data in this thesis do not support the notion that anthocyanins or their metabolites improve lipid/lipoprotein profiles and biomarkers of HDL function.

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ACAT1	acyl coenzyme A:cholesterol acyltransferase-1
2-HQ	2-hydroxyquinline
3MethGA	3-*O*- Methylgallic acid
4HBA	4-hydroxybenzaldehyde
4MethGA	4-*O*-Methylgallic acid
5HFA	5-hydroxy ferulic acid
ABCA1	ATP-Binding Cassette transporter type A1
ABCG1	ATP-Binding Cassette transporter type G1
Ac-LDL	Acetylated-LDL
ANCs + Flavonols	Tomatoes that contain a mixture of anthocyanins and flavonols
Apo A1	apolipoprotein A1
Apo B	apolipoprotein B
C3G	Cyanidin-3-glucoside
c-AMP	Cyclic adenosine monophosphate
CE	Cholesteryl esters
CEC	Cholesterol efflux capacity
CETP	Cholesteryl-ester transfer protein
CHD	Coronary heart disease
COX-2	Cyclooxygenase
CPM	Counts per million
CVD	Cardiovascular disease
D3G	Delphinidin-3-glucoside
DAD	diode array detection
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DTNB	5,5′-Dithiobis (2-nitrobenzoic acid)
EMT	Epithelial-mesenchymal transition

eNO	endothelium-derived nitric oxide
FBS	Fetal bovine serum (FBS)
FC	Free cholesteryl
FW	Fresh weight
GA-3-GlcA	gallic acid-3- glucuronide
GA-4-GlcA	gallic acid-4-glucuronide
GPO-POD	Glycerol-3-phosphate oxidase-Peroxidase
GSEA	Gene set enrichment analysis
HDL	High-density lipoprotein
HMG-Co-A	Hydroxymethylglutaryl-CoA
HPLC/MS	High performance liquid chromatography/Mass spectrometry
HUVECs	Human umbilical vein endothelial cells
IL	Interleukin
JAMs	Junction adhesion molecules
LCAT	Lecithin-cholesterol acyl transferase
LDL	Low-density lipoprotein
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LXR	Liver X receptor
MI	myocardial infraction
MsigDB	The Molecular Signatures Database hallmark
NGS	Next generation sequencing
Ox-LDL	Oxidized low-density lipoprotein
PCA	Protocatechuic acid
PCA-3-GlcA	PCA-3-glucurunide
PCA-3-Sulph	PCA-3-sulphate
PCA-4-GlcA	PCA-4-glucuronide
PCA-4-Sulph	PCA-4-sulphate
PGA	Phloroglucinaldehyde
PMA	phorbol 12-myristate 13-acetate

PON-QQ	PON1 phenotype QQ
PON-RR	PON1 phenotype RR
PPARs	Peroxisome proliferator-activated receptors
PTGS	prostaglandin-endoperoxide synthase
PSP	Purple sweet potatoes
QIB	Quadram Institute Bioscience
RCT	Reverse cholesterol transport
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RNA seq	RNA sequencing
rRNA	Ribosomal RNA
RXR	Retinoic X receptor (RXR)
SNP	Single nucleotide polymorphism
SR	Scavenger receptor
TBBL	5-(Thiobutyl) butyrolactone
TNC	Tenascin
TNFα	Tumour necrosis factor alpha
VEGF	vascular endothelial growth factor

Symbols

%	Percent
ºC	Degrees celsius
CO_2	Carbon dioxide
g	Gram
h	Hour
kg	Kilogram
kg/m2	Kilogram per square meter
m/s	Pulse wave velocity (meter per second)
mg	Milligram
mg/day	Milligram per day
mg/dL	Milligrams per deciliter
mg/kg	Milligram per kilogram
mg/L	Milligram per litre
min	Minute
mIU/L	Milli-international units per litre
ml	Millilitre
ml/min	Millilitre per minute
mm	Millimetre
mM	Millimolar
mmHg	Millimeter of mercury
mmol/L	Millimole per litre
ng	Nanogram
ng/ml	Nanogram per millilitre
nM	Nanomolar
v/v	Volume per volume
w/w	Weight per weight
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
µM	Micromolar

List of publications and presentations

Peer-reviewed Papers

H.T. Aboufarrag, W. Hollands, A. Blair, N. Perez-Moral and P. Kroon. The effect of anthocyanin consumption on the lipid/lipoprotein profiles, HDL function and paraoxonase 1 (PON1) activities in relation to PON1 genotype in humans: Randomized placebo-controlled crossover trial. **In preparation**

H.T. Aboufarrag, M. Winterbone, P. Troncoso Rey, P. Needs and P. Kroon. The effects of anthocyanins and their metabolites on foam cell formation and other atherosclerotic-related pathways. **In preparation**

HassanT. Aboufarrag, Paul W. Needs, Gerlad Rimbach and Paul A. Kroon (2019). The Effects of Anthocyanins and Their Microbial Metabolites on the Expression and Enzyme Activities of Paraoxonase 1, an Important Marker of HDL Function**.** Nutrients 11(12), 2872**.** https://doi.org/10.3390/nu11122872. See appendix 7.

Karen F Chambers, Priscilla Day, **Hassan T Aboufarrag** and Paul A. Kroon (2019). Polyphenol effects on cholesterol metabolism via bile acid biosynthesis, CYP7A1 and the gut microbiota: A review. Nutrients 11(11) 2588. https://doi.org/10.3390/nu11112588. See appendix 8.

Posters

Hassan T. Aboufarrag and Paul A. Kroon. Effects of anthocyanin metabolites on markers of reverse cholesterol transport. 1st International conference on food bioactives and health, Norwich, UK, September 12-14.

Hassan T. Aboufarrag and Paul A. Kroon. Effects of anthocyanin metabolites on markers of reverse cholesterol transport. QIB Student Science Showcase, June 2017.

Oral presentations

Hassan T. Aboufarrag (2017) Effects of anthocyanins and their metabolites on markers of reverse cholesterol transport. Knowledge exchange trip to the German Institute of Human Nutrition, Potsdam, Germany, August 11-12

Hassan T. Aboufarrag (2017) Interactions between dietary anthocyanins, cholesterol metabolism and quality, and PON1 genotype. Wageningen University & Research Student visit to QIB. Norwich, UK, October 20.

Hassan T. Aboufarrag (2018) Interactions between anthocyanin microbial metabolism, cholesterol metabolism and PON1 genotype. QIB Coffee Break Science, Norwich, UK, August 24 (DATE).

Hassan T. Aboufarrag (2019) Interactions between anthocyanin microbial metabolites, HDL function and PON1 genotype. NUGO week, 16th edition. Bern, Switzerland, September 9-12.

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CHAPTER ONE:

General introduction

1.1. Structure of the thesis

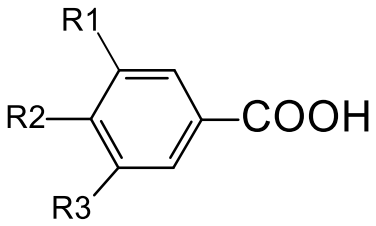
The data presented in this thesis is about the underlying molecular mechanisms by which anthocyanins and their metabolites would provide atheroprotective effects with the main focus on lipid metabolism using in-vitro and in-vivo models. Chapter one introduces anthocyanins, their bioavailability and metabolism, their related health benefits. It defines the gaps in knowledge which need to be investigated and details the aims and objectives of this thesis. Chapters two and three present the results of in-vitro models that were used to investigate the effects of anthocyanins and their selected metabolites on markers related to reverse cholesterol transport and HDL function. Chapter four presents RNA transcriptomic analysis as a non-targeted in-vitro approach to identify other possible atherosclerosis-linked genes and the pathways affected by the anthocyanins. Chapter five, on the other hand, presents the results of a human intervention trial conducted to investigate the effects of two different types of purified anthocyanin extracts on lipid/lipoprotein profiles and quality, and markers of HDL function.

1.2. What are (poly)phenols

(Poly)phenols are a diverse group of phytochemicals containing single or multiple phenol rings [1]. They are distributed in the plant kingdom, including fruits and vegetables [2]–[5]. (Poly)phenols are secondary metabolites arising from the shikimate and acetate-malonate (malonyl-CoA) biosynthetic pathways in plants [6]–[8]. There are more than 8,000 phenolics and (poly)phenols are classified into subgroups according to their chemical structure. Flavonoids, phenolic acids, lignans and stilbenes are the main subgroups of (poly)phenols [9], [10]. The flavonoids subgroup can be further classified into anthocyanins, flavan-3-ols, flavanones, flavonols, flavones and isoflavones [11]. Fig 1.1 and 1.2 demonstrate the main (poly)phenols and flavonoids classes. Although, (poly)phenols are found in all plant tissues, they are usually most concentrated in fruits and leaves [12]. The main sources of (poly)phenols in food are fruits, vegetables, tea, wine, beverages, chocolate and other cocoa products [11], [13], [14]. The main focus of my project was anthocyanins; thus this class will be described in more detail.

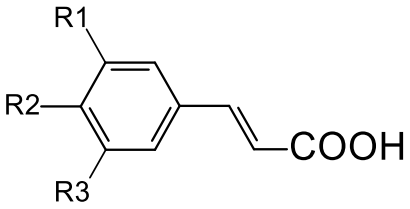
1.3. Anthocyanins, what they are?

Anthocyanins are a subgroup of flavonoid-type bioactive compounds that belong to the overarching phytochemical group called (poly)phenols [15]. Anthocyanins are one of the most important plant pigments that are responsible for red, pink, violet and blue colours of fruits, seeds, flowers and other parts of many plants [16], [17].



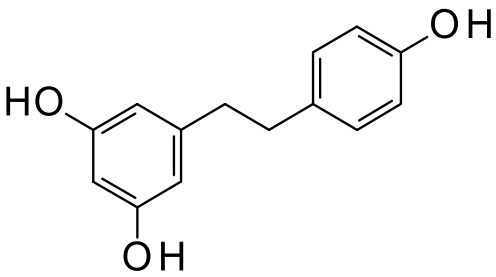
Hydroxybenzoic acid

R1=H, R2=OH, R3=OH: protocatechuic acid



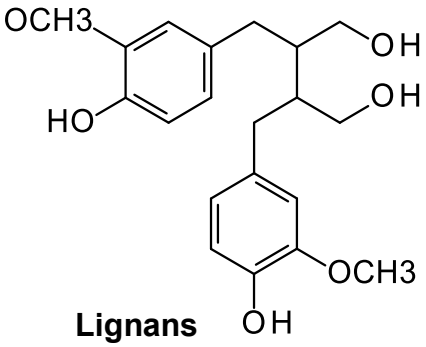
Hydroxycinnamic acid

R1=H, R2=OH, R3=OCH_3: ferulic acid



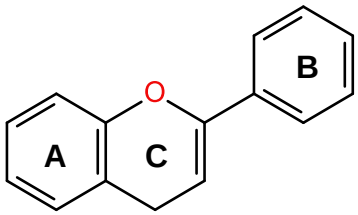
Stilbenes

Resveratrol



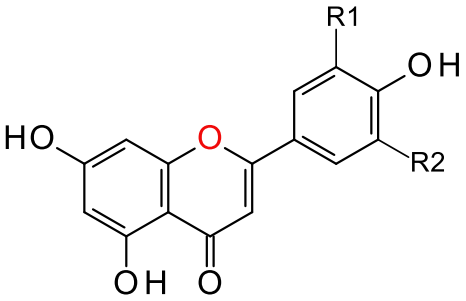
Lignans

Secoisolariciresinol



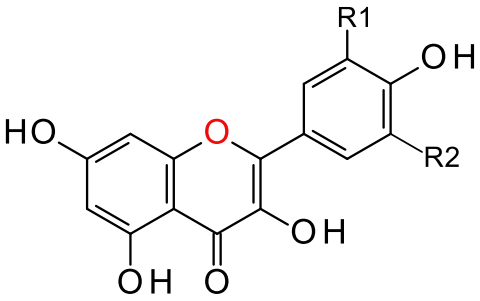
Flavonoids backbone

Figure 1. 1: Chemical structure of (poly)phenols.



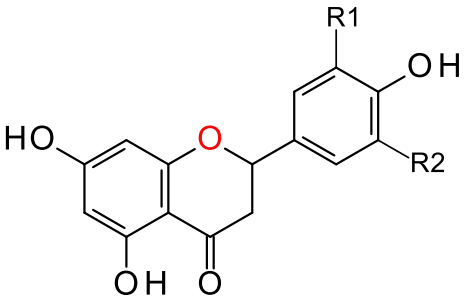
Flavones

R1=R2=H: Apigenin



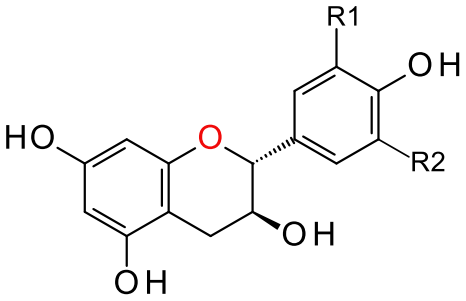
Flavonols

R1=OH, R2=H: Quercetin



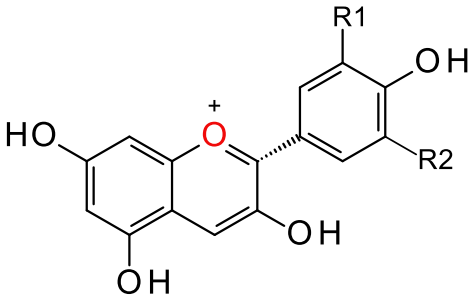
Flavanones

R1=R2=H: Naringenin



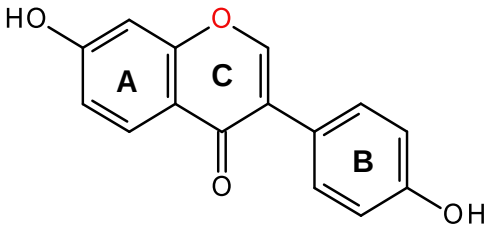
Flavan-3-ols

R1=OH, R2=H: (+)-Catechin



Anthocyanidins

R1=OH, R2=H: Cyanidin



Isoflavonoids

Daidzein

Figure 1. 2: Chemical structure of flavonoids.

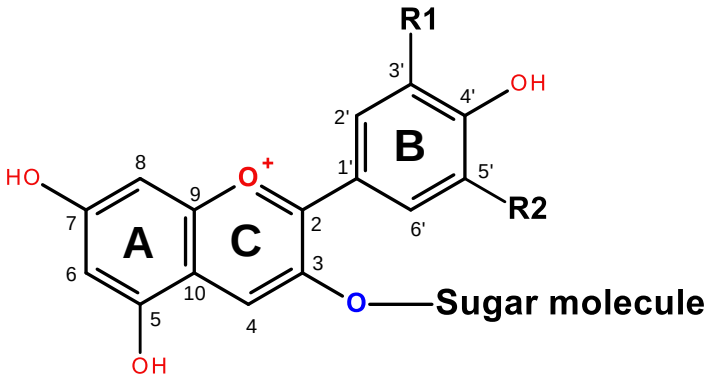
The word anthocyanin is derived from the two Greek words anthos (= flower) and kianos (= blue) [18]. Their water-soluble nature makes them the most commonly used colourants in the food industry [19]. Anthocyanins are predominately present in the skin of fruits, although in berries, they are found in the skin and flesh [20]. Anthocyanins are involved in many functional roles in plants such as attracting the pollinators and seed dispersing animals ; and protecting against UV light and other environmental stress factors [21], [22]. Anthocyanins have two absorption peaks, one in the UV range between 270 to 280 nm and another which is in the visible region between 465 to 550 nm [23], [24].

1.3.1. Chemical structure of anthocyanins

Chemically, anthocyanins consist of an aglycon called anthocyanidin bound to several sugar moieties [25]. Anthocyanidins are polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium and have the same C6-C3-C6 general backbone structure of flavonoids [26]. Anthocyanidins consist of three aromatic rings; the A-, B- and C-rings (Fig 1.3) [27]. The A-ring and C ring are fused together and bonded by a carbon–carbon bond to the B-ring [28]. To form the glycoside form (anthocyanins), a sugar molecule glycosidically linked to a hydroxyl group on the anthocyanidin (aglycon), at the 3-position on the C-ring or the 5, 7-position on the A-ring [29]. The sugar molecules are usually glucose, rhamnose, galactose or arabinose and are attached as mono-, di-, or trisaccharide forms [30].

1.3.2. Types and distribution of anthocyanins

There are many types of anthocyanins in nature. To date, more than 500 anthocyanins and 17 anthocyanidins have been reported and the number is still rising [31]. The main differences between them are the number and positions of hydroxyl and methoxyl groups attached to anthocyanidin structure; the nature and the number of the sugar moiety bonded with anthocyanidin; and the position of these bonds [32], [33]. Among the 17 anthocyanidins present in nature, only six are common in food; cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin (see Fig 1.3) [34]. The distribution of these aglycons is: Cyanidin 50%, delphinidin 12%, pelargonidin 12%, peonidin 12%, petunidin 7% and malvidin 7% [35]. These six aglycons differ chemically at the 3` and 5` position of the B-ring (see Fig 1.3).



Anthocyanidin	R1	R2	Colour
Pelargonidin (Pg)	H	H	Orange
Cyanidin (Cy)	OH	H	Orange-red
Delphinidin (Dp)	OH	OH	Blue-red
Peonidin (Pn)	OCH_3	H	Orange-Red
Petunidin (Pt)	OCH_3	OH	Blue-red
Malvidin (Mv)	OCH_3	OCH_3	Blue-red

Figure 1. 3: Chemical structures of anthocyanins

Anthocyanins also differ in colour according to the substitution at the B-ring with colour goes toward more bluish if more hydroxyl groups are present or goes toward redness if more methoxyl groups are present [36], [37]**.** The non-methylated anthocyanidins (cyanidin, delphinidin, and pelargonidin) are found in 80% of pigmented leaves, 69% in fruits and 50% in flowers. Moreover, 3-monoglycosides, 3-diglycosides, and 3,5-diglycosides are most commonly sugars bound to anthocyanidins. Furthermore, 3-monoglycosides occurrence is 2.5 times higher than other glycosides and cyanidin-3-glucoside is the most abundant anthocyanin type found in nature [17]**.**

1.3.3. Anthocyanins sources and daily intake

Fruits including red and blue berries, blackcurrant, blood orange and red grape, vegetables with coloured skin, as well as red wine, are the most common sources of anthocyanins amongst a long list of other human dietary sources [10].
Within different plants, anthocyanins content ranges from 0.25 mg/100 g Fresh Weight (FW) in pear to 500 mg/100 g FW in blueberries [38]. Also, anthocyanin levels noticeably vary in the same plant according to the cultivar, season and agronomic factors [39]. In fruit, the major sources of anthocyanin were found to be elderberry, chokeberry, bilberry**,** raspberry**,** blueberry, blackberry and red grape, while, purple corn and red cabbage are the main anthocyanin sources in vegetables. Table 1.1 shows the total anthocyanin content of some fruits and vegetables.

Not only the anthocyanin content but also the anthocyanin type is quite different among food sources. For instance, black raspberry contain the highest levels of cyanidin-type anthocyanins (669 mg/ 100 g FW), while, black currant contain the highest levels of delphinidin-type anthocyanins (333 mg/100g FW) [40]**.**

The average daily intake of anthocyanins strongly depends on various factors like the dietary habits of a population, season and culture. Therefore, there are big differences in the reported anthocyanins intake [40] . In Europe, the mean intake of anthocyanidins for men ranged from 19.8 mg/day (Netherlands) to 64.9 mg/day (Italy), whereas for women the range was 18.4 mg/day (Spain) to 44.1 mg/day (Italy). 61 % of habitual intake comes from fruit, while wine contributed to 14.4-24.5% of intake. Higher consumption by Italians may result from their Mediterranean diet, which includes berries, and other red and blue-coloured fruits, and red wine [15]. In Finland, on the other hand, the daily intake of anthocyanins was suggested to be 82 mg/day [18]. The anthocyanins daily intake in USA was estimated to be 12.5 mg/day based on the concentration data and updated food intake data from NHANES 2001−2002 [40]. On the other hand, some reports suggested that the daily consumption of

anthocyanins is anywhere from 3 to 215 mg/day as previous data were derived from questionnaires and dietary recall which often underestimate intakes. Doses ranging from 400 to 500 mg of anthocyanins can easily be obtained from one serving of berries and some juices [41].

Table 1. 1: Anthocyanins content in common dietary sources

Food	Total anthocyanins content (mg/100 g fresh weight)	Reference
Elderberry	200-1816	[27], [40]
Chokeberry	410-1480	[37]
Red grape	30-750	[42]
Bilberry	300-698	[43]–[45]
Raspberry	20-687	[46], [47]
Blueberry	25-495	[48]–[50]
Blackberry	82.5-325.9	[51], [52]
Plum	2-25	[26]
Strawberry	13-36	[51], [53]
purple corn	1642	[54]
Eggplant	750	[55]
Cabbage	322	[35]
Black rice	23-207	[56]–[60]
Port Wine	14-110 (mg/100mL)	[61]
Red wine	16.4-35 (mg/100mL)	[62], [63]
Pomegranate (Juice)	44 (mg/100mL)	[64], [65]

1.4. Bioavailability and metabolism of anthocyanins

Bioactive compounds such as anthocyanins need to be bioavailable in order to exert their activity. Therefore, it is not only important to know the concentration of nutrients in food, but it is even more important to know the fate of these nutrients in the body and how much is bioavailable. The term “bioavailability” means the proportion of the nutrient that is digested, absorbed and reaches the circulation intact after oral administration. Other definitions included both intact and metabolised forms [38].

The biological activities of anthocyanins are strongly linked to their absorption and metabolism in vivo. Anthocyanins can be rapidly absorbed and detected in circulation within minutes of consumption in their parent forms as a result of absorption through gastrointestinal tract, in particular from the small intestine [66], [67]. Anthocyanins required 0.5 to 2 h to reach C_{max} in plasma which is far faster than other flavonoids [27]. Some researchers suggested that the anthocyanin metabolism start in the oral cavity as a result of interaction with salivary proteins and digestive enzymes [19], [46]. In saliva samples that collected from healthy volunteers following a human intervention with black raspberry, several derivatives of black raspberry anthocyanins were detected including their hydrolysed from (aglycon), glucuronidated conjugates and few quantities of protocatechuic acid resulting from the activity of β-glycosidase derived from bacteria and oral epithelial cells or as a result of oral microbiota [46]. However, there is no information about the absorption of anthocyanins in the oral cavity [18].

In the stomach, given the quick appearance of anthocyanin in the circulation after ingestion (approximately 30 min), it was suggested that anthocyanins able to cross the gastric system and reach to the circulation [19], [27]. This suggestion was supported with in vivo and in situ models (using mainly rat model) and reported that about 20% of the total absorbed anthocyanins were absorbed from the stomach in their intact forms [68]–[70]. The reason could be that the acidic gastric juice increased the stability of glycoside forms [52], [69]. In fact, there is ambiguous information whether the stomach is only an absorption organ or metabolizing organ and the mechanism of anthocyanin gastric absorption remains unknown [19]. Although the reported absorption of anthocyanin in stomach, the vast majority of animal and human studies suggest that the intestine is the major site of anthocyanin absorption [71].

After passing the stomach, anthocyanins arrive in the small and large intestine. Following entry into the small intestine where more basic conditions are present, the intact as well as methylated and conjugated forms of anthocyanins were quickly

absorbed [52]. The absorption is associated with the action of several hydrolysis releasing enzymes such as lactase phlorizin hydrolase in the brush border of the small intestine. The aglycones usually undergo phase II transformation in the enterocytes, producing sulphate, glucuronides, and/or methyl forms through the respective action of sulfotransferases, uridine-5`-diphosphate glucuronosyltransferases and catechol-O-methyltransferases [71]. Unabsorbed anthocyanins then reach the colon where substantial structural modification takes place including ring fusion and phase 11 metabolism leading to the production of many smaller molecules such as phenolic acids and their conjugates. Microbiota most likely play an essential role in this biotransformation [15].

In general, the absorption of anthocyanins is dependent on their molecular structure. In human and rat, the glycosylated forms are less absorbed than the hydrolysed forms [72]. Probably because the glycosylated forms have higher molecular weight. Moreover, anthocyanins carrying the same sugar moiety were absorbed as following: delphinidin > cyanidin > malvidin. This may be a result of the greater number of hydroxyl groups in delphinidin or the greater hydrophobic nature of malvidin. Similarly, anthocyanins with the same aglycone type were absorbed in the order: galactoside > glucoside > arabinoside [72].

Comparing the amount of the intact anthocyanins absorbed and excreted in urine to the total ingested doses; anthocyanins appear to have low bioavailability, with a recovery of < 1% of consumed doses of anthocyanins in the majority of studies [73]. Manach *et al.* (2005) reviewed 97 human interventions that investigated the kinetics and extent of polyphenol absorption [74]. They found that the concentration of anthocyanins measured in plasma were ranged from 10-50 nmol/L and the mean time to reach C_{max} was 1.5 h (range: 0.75-4 h) for plasma and 2.5 h for urine. They also found that concentration of anthocyanins excreted in urine were 0.1% of the intake. Other reported concentrations of anthocyanins 1 – 100 nmol/L in plasma and urine following consumption of berries and grapes [27]. Furthermore, Czank *et al.* (2013) found that only 36.47 ng of cyanidin-3-glucoside were recovered in plasma, urine and faeces after consumption of 500 mg of ^{13}C-labelled cyanidin-3-glucoside [75]. Table 1.2 adapted from Kay (2006) [76] summarises the measured bioavailability of intact anthocyanins in human.

The most pressing issue is how to explain the high bioactivity of anthocyanin despite the reported low recovery (bioavailability). Some researcher suggest that the bioavailability of anthocyanins was underestimated [77]. The reason for this suggestion was that the main methods used in analysis were based on measuring

anthocyanins in their coloured acidified forms (flavylium ions) and therefore, other colourless forms have not been quantified [77]. However, this suggestion doesn't explain entirely the low recovery. Other suggest that the reason of the reported low bioavailability of anthocyanins is that anthocyanins expose to extensive metabolism and biotransformation, and therefore, the concentration of the intact forms are very low [19]. In fact, the degradation of anthocyanins into smaller molecules such as phenolic acid is the most likely event. There are several reports supporting this notion. For example, Prior and Wu (2006) reported that 60–90% of the anthocyanins disappeared from the gastrointestinal tract within 4 h after a meal; and just very low concentrations of intact compounds were observed, suggesting that anthocyanins transformed into other forms [27]. In addition, 30 to 44% of blood orange anthocyanins were found as protocatechuic acid (PCA) in plasma supporting suggesting that anthocyanin underwent extensive metabolism [78]. This notion was confirmed using use of isotopically labelled anthocyanins. Czank, *et al.* (2013) [75] and de Ferrars, *et al.* (2014) [73] confirmed this notion when they identified a substantial number and amount of breakdown products derived from ^{13}C-labelled anthocyanins and about 43% of the ^{13}C dose was recovered.

Table 1. 2: Bioavailability of anthocyanins following single dose administration in humans*

Source	Period (h)	Dose (mg)	Urinary recovery (%)	C_{max} (nmol/l)	t_{max} (h)	$t_{1/2}$ (h)	Reference
Chokeberry	24	721	0·15	96·1	2·8	1·5	[79]
Hibiscus extract	7	147	0·018	7·6	1·5	2·6	[80]
Red grape juice	7	283	0·23	222·7	0·5	1·8	[81]
Red wine	7	280	0·18	95·5	1·5	2·0	[81]
Blackcurrant	7	145	0·04	—	—	1·7	[82]
Elderberry	7	147	0·37	—	—	1·7	[82]
Chokeberry	24	1300	0·048	592	—	—	[83]
Strawberries	24	77	1·9	—	—	—	[84]
Blackcurrant	4	716	0·05	35·6	0·7	—	[85]
Blueberry	4	1200	0·003	29·2	4·0	—	[86]
Blackcurrant	7	1000	0·039	—	—	—	[87]
Elderberry	24	720	0·08	97·4	—	—	[88]
Blueberry	6	690	0·004	—	—	—	[88]
Elderberry	—	500	0·05	—	—	—	[89]
Blackcurrant	5	153	0·03	—	—	—	[90]

- *

Cmax, maximum concentration derived from serum or plasma data; tmax, time to reach maximum concentration derived from serum or plasma data; t1/2, half-life of elimination. Table 1.2 is adapted from Kay (2006) [76]

Based on the reported fact that anthocyanins undergo extensive metabolism and few quantities of intact form were detected in human fluids, a significant question remains about the biologically active form of anthocyanins. Either anthocyanin parent compounds are very active at low nanomolar concentration which is not reported anywhere; or their metabolites which are found in 6 to 42-fold higher abundance are responsible for the biological activity of anthocyanins. Therefore, the relative biological activity of the parent compounds and their metabolites remains to be studied.

1.4.1. Metabolism of cyanidin-3-glucoside

Czank *et al*. (2013) studied the metabolism of ^{13}C-labelled cyanidin-3-glucoside in human participants and found that the major metabolites in serum were phase II conjugates of PCA, ferulic acid, and hippuric acid. The maximum concentration for phase II conjugates of PCA was 2.35 µM, while the maximum concentration of cyanidin-3-glucoside was 0.14 µM [75]. Similar findings were observed when the metabolism of ^{13}C-labelled cyanidin-3-glucoside was studied by de Ferrars *et al.* (2014) [73]. A total 35 of metabolites were identified, of which 17 were found in the plasma, 31 in the urine, and 28 in the faeces. In the serum, PCA, 13 derivatives of PCA, phloroglucinaldehyde (PGA) and one derivative of PGA were identified. The concentration ranged from 11 nM for PCA-3- glucuronide to 1962 nM for hippuric acid, while the concentration of the parent compound (cyanidin-3 glucoside) reached 141 nM. Eight sulphated and glucuronidated forms of PCA and methylated PCA (vanillic acid) were identified within the serum. Apart from hippuric acid, vanillic acid (1845 nM), ferulic acid (827 nM), 4-hydroxybenzaldehyde (667 nM), vanillic acid sulphate (430 nM) and PCA sulphate (157 nM) were the predominant metabolites present in serum. In addition, the methylated form; peonidin-3-glucoside as well as the glucuronide form were also detected after consumption of cyanidin-3-glucoside, although in low concentrations [73].

1.4.2. Metabolisms of other anthocyanin types

The metabolism of other anthocyanin types such as delphinidin and pelargonidin type has also been studied. However, anthocyanin-rich dietary sources were used instead of pure or isotope labelled compounds.

Mueller *et al.* 2017 [91] studied the metabolism of bilberry extract powder that contain mainly trihydroxylated-B ring anthocyanins as well as cyanidin-3-glucoside in humans with and without colon. They found that the main metabolites in ileostomy fluid, which represent the compounds available to the colon from the small intestine, were PCA,

gallic acid and PGA. Gallic acid was suggested to originate from B-ring of delphinidin glycosides. On the other hand, syringic acid (a partially methylated form of gallic acid) and vanillic acid (a methylated form of PCA) were the predominant metabolites in the plasma of healthy subjects, with concentrations represent 45 and 24.6 % of total metabolites, respectively. It was difficult to decide whether syringic acid and vanillic acid derived from the methylation of gallic acid and PCA; or if they originated from methylated anthocyanins that present in bilberry extract such as petunidin, peonidin and malvidin glycosides.

Azzini *et al.* (2010) studied the metabolism of pelargonidin-3-glucoside in humans by using strawberry fruit as a dietary source [92]. In serum, 4-hydroxybenzoic acid as well as coumaric acid were identified, while 4-hydroxybenzoic acid was described as the major metabolite. The concentration of 4-hydroxybenzoic acid and coumaric acid were 2.5 and 0.37 µM, respectively. However, a low amount of the parent compounds, both as aglycones and glucuronide conjugates were identified in urine. Moreover, Mullen *et al.* (2008) identified pelargonidin-O-glucuronide at a maximum concentration of 274 nM, together with traces of pelargonidin-3-glucoside in human plasma after strawberry consumption as a source of pelargonidin-3-glucoside [93]**.**

In addition to that, there are several recent human interventions that measured the anthocyanin metabolites. For instance, a recent human intervention detected about 63 metabolites in plasma following the consumption of aronia crude extract. Most of the detected metabolites were present in nanomolar concentrations except for hippuric acid, benzoic acid, phenylacetic acid, and 3-(4-hydroxyphenyl) propionic acid, which were present at micromolar concentrations [94].

Overall, the concentration and stability of anthocyanin metabolites in biological fluid is higher than the parent compounds suggesting that high bioactivity of anthocyanins is more likely to be mediated by the metabolites. However, the biological activity of these metabolites, which compounds are more beneficial and at which concentrations is yet to be investigated. In addition, these metabolites are found in mixtures and the interaction between these metabolites is most likely to take place, thereby it needs to be examined.

1.5. Anthocyanins and cardiovascular disease (CVD)

Growing evidence suggests that diets rich in fruits are one of the most important factors for reducing the rate of some chronic diseases such as CVD. This is most likely because of the abundance of bioactives such as (poly)phenols including anthocyanins in these foods [95]. Numerous studies highlight the beneficial effect of

flavonoids, anthocyanins in particular, as key constituents in lowering CVD risk factors [15]; and therefore the current project focused on the relation between anthocyanins and the reduction in CVD risk.

CVD is the biggest cause of mortality worldwide [96], [97]. It accounts for 30 % of all deaths worldwide [98] and the number of people dying from CVD is expected to reach to 23.6 million in 2030 [99]. In UK, around 7.4 million people live with heart and circulatory disease and around 168 thousand died from CVD in 2017 which account for 28% of all deaths in UK. The total annual healthcare cost of heart and circulatory disease in the UK was estimated as £9 billion [100]. Although CVD term involves all disease affecting heart and/or blood vessels, atherosclerosis is the main CVD type [101] and thereby, atherosclerosis will be the main focus in this thesis.

1.5.1. Pathobiology of atherosclerosis

Atherosclerosis is a chronic, progressive and inflammatory disease in which the arteries become hard and narrow as a result of being clogged up by fatty substances called plaques or atheroma [102]. The key cellular players in the development of this disease are endothelial cells, leukocytes, platelets and intimal smooth muscle [103], [104]. Atherosclerosis is associated with principal CVD such as coronary heart disease (CHD), carotid artery disease, and peripheral arterial disease which lead to a decrease of blood flow and oxygen supply and which may result in acute cardiovascular events such as a heart attack or stroke and can eventually lead to death [105]–[108]. Elevated cholesterol level is the main CVD risk that drives the development of atherosclerosis; however, other risk factors such as hypertension, diabetes, smoking, male gender, and inflammatory markers appear to accelerate the progress of the disease [109].

Atherosclerosis begins as an inflammatory response to endothelial injury caused by oxidized low-density lipoprotein (ox-LDL) within the endothelium. Ox-LDL accumulates as a result of high low-density lipoprotein (LDL) and cholesterol levels in blood. This injury triggers the monocytes, T-cells, chemokines and membrane adhesion molecules; and attracts immune cells to the site. The adhesion molecules then change the endothelial cells permeability and facilitate the migration of monocyte into the injury site. In response to potent cytokines and mitogens, monocyte mature into macrophages which then scavenge the ox-LDL and dead cells through phagocytosis forming what are called cholesterol-laden macrophages or foam cells, the hall mark of atherosclerosis.

The accumulation of living and dead foam cells, endothelial cells and smooth muscle cells increase the vessel thickness and decrease the lumen. At a later stage, the plaque can be ruptured, close up the lumen, compromise the blood flow and reduce or block the flow of blood to the organs which may lead to death [95], [110]–[113].

Healthy diet (e.g. a diet rich in fruits and vegetables) and healthy lifestyle (e.g. physical activity, no smoking) are the most important protective factors against atherosclerosis. In addition, high-density lipoprotein (HDL) and its major apolipoprotein (Apo) '(Apo A1)' play a significant role in attenuation of atherosclerosis by reducing the atherogenic modification of LDL and promoting the process of reverse cholesterol transport (RCT) which slows down the foam cell formation by lowering cholesterol accumulation [114], [115].

Promotion of RCT is the main mechanism which lowers the cholesterol accumulation in foam cells, and it may be the target of anthocyanins treatments. Therefore, more studies are required to understand the interaction between anthocyanins and/or their metabolites and RCT and how anthocyanins treatments may contribute to the enhancement of RCT and HDL function. For more details about the role of RCT in foam cell formation and atherosclerosis development, see sections 2.2.

Many epidemiological, meta-analysis and clinical studies suggest that the consumption of anthocyanins-rich fruit and vegetables are associated with reduced atherosclerosis risk.

1.5.2. Epidemiological association between anthocyanins and lowering CVD risk.

Several epidemiological studies have studied the association between food rich in anthocyanins (such as berries and red wine) and CVD outcomes or biomarkers [116]. Cassidy *et al.* (2016) recently found that anthocyanins intake is inversely associated with nonfatal myocardial infraction (MI) but not with fatal MI in a study that followed 43,380 healthy men for 24 years (Health Professionals Follow-Up Study [117]). The association was stronger in normotensive participants. Another recent study followed 93,600 women aged 25-42 years for 18 years. An inverse association between higher intake of anthocyanins and risk of MI was observed [118]. In this study, the reduction in the risk was 32% comparing the participants who consume the highest versus the lowest quantity of anthocyanins. Median intakes in this study were 12 mg/day anthocyanins with a continual dose-response at higher levels of intakes with 17% reduction in MI for every 15 mg/day increase in anthocyanins intake in the multivariate model [118]. In another study by Cassidy *et al.* (2011), it was found that anthocyanin

consumption (predominantly from blueberries and strawberries) was correlated with an 8% reduction in risk of hypertension in a study which followed 87,272 individuals for 14 years. The reduction increased to 12% in participants ≤ 60 year of age [119]. Furthermore, the association between anthocyanins intake and CVD mortality was investigated. The Iowa Women's Health study which followed 34,489 postmenopausal women for 16 years found an association with anthocyanin intake and reduced risk of CHD and CVD-related mortality [120]. Anthocyanin intake reduced the risk of CHD, CVD and CVD-related mortality by 12, 9 and 10%, respectively. The significant reduction was correlated with the consumption of one portion of strawberry and blueberries once a week [120]. Similarly, McCullough *et al.* (2012) found that high intake of anthocyanidins (the aglycon form of anthocyanins) and other classes of flavonoids were associated with a lower risk of fatal CVD. The risk reduced by 18% in high intake compared to the low intake of anthocyanidins and flavonoids [121].

Moreover, several epidemiological studies provide mechanistic support for the association between high intake of anthocyanins and observed decrease in CHD. For instance, higher intake of anthocyanins improved arterial stiffness and blood pressure [119]. Moreover, high intake of anthocyanins was correlated with a 4 mmHg decrease in central systolic blood pressure which is similar to those who stopped smoking, a 2.3 mmHg decrease in medium arterial pressure and a 0.4 m/s decrease in pulsive wave velocity [122]. In a recent study, Jennings *et al.* (2014) found that higher habitual intake of anthocyanins resulted in a 0.7 mIU/L reduction in insulin concentrations in blood which is similar to the effect of 1 h/day walking and a low-fat diet [123].

In contrast, some other epidemiological studies found no association between anthocyanin intake and CVD risk factors. For instance, Mursu *et al.* (2008) found no association with high anthocyanins intakes and CVD mortality after following Finnish men aged 42-60 years over 15 year [124]. Additionally, there was no significant association with flavonoids consumption and CVD risk factor in a study with 38,445 healthy female [125]. Moreover, Cassidy *et al.* (2012) found no association between anthocyanin intake and ischemic control after following 69,622 women for 14 year from the Nurses' Health Study, although, a non-significant modest inverse association between a higher intake of flavones and anthocyanins and risk of total and ischemic stroke was observed [126]. The discrepancies between studies may result from a failure to accurately assess intake in human populations due to insufficient information from the questionnaires used in these studies, limited information in food composition

databases and/or the nature of the studied population, e.g. populations with a high baseline intake showed no association [116].

1.5.3. Evidence from meta-analysis studies

There are several systematic reviews and meta-analysis studies that reported an association with the consumption of anthocyanins and reduction in CVD risk factors. In a very recent systematic review and meta-analysis of 17 randomized clinical trials that reported the effect of crude and purified anthocyanins on lipid profile and inflammatory markers, fifteen studies reported significant in biomarkers related to CVD [127]. Except for total cholesterol, all other lipid profile parameters such as LDL, HDL-cholesterol and triglyceride were positively influenced by anthocyanin treatment. In the same study, a significant reduction in TNF-α was also reported after anthocyanin consumption [127]. In another recent meta-analysis of 45 randomized clinical trials that included 2,053 patients has reported a correlation between the intake of berries and a reduction in LDL-cholesterol, triglyceride and blood pressure and increase in HDL-cholesterol level [128]. The study presumed that the dual beneficial effect of consumption of berries on lowering LDL and increasing HDL is linked to the CETP inhibition [128]. Additionally, a meta-analysis study that included 50 prospective cohort trials reported a relationship between fruit and vegetable intake and a reduction in CVD risk factors. The dose-response analysis showed that people who consumed 800 g per day of fruit and vegetables had the lowest risk of CVD [129]. On the other hand, a recent systematic review and meta-analysis of nineteen randomized clinical trials reported no significant effects of anthocyanin supplementation on reducing LDL-cholesterol and increasing HDL-cholesterol in the pooled results of all nineteen trials [130]. However, when the authors sub-grouped the trials by dose and duration, they reported a positive influence of anthocyanin intake on total and LDL-cholesterol at doses ≥ 300 mg/day for more than 12 weeks duration[130]

1.5.4. Evidence from dietary intervention studies

Several randomized clinical trials have examined the effect of consumption of anthocyanin on several CVD biomarkers including lipid profile, blood pressure, endothelial dysfunction, inflammation, arterial stiffness, and platelet activation.

1.5.4.1. Effect of anthocyanin consumption on blood pressure

In a very recent study, Rodriguez-Mateos *et al.* (2019) found that consumption of 500 ml of blueberry drink that contain 150 mg anthocyanins for more than 28 days

significantly reduced 24h ambulatory systolic blood pressure by -5.6 mmHg, although no significant difference in diastolic blood pressure was observed [131]. In another recent study, a significant decrease in diastolic blood pressure was observed after consumption of 500 mL anthocyanin-rich orange juice corresponding to 250 mg/day anthocyanins for 16 weeks [132]. Moreover, Aviram *et al.* (2001) observed a significant 5% reduction in systolic blood pressure after consumption of pomegranate juice for 14 days in hypertensive individuals. In the same study, the reduction was increased to 21% in systolic blood pressure after one-year consumption of pomegranate juice in larger group of participants with asymptomatic severe carotid artery stenosis [133]. However, 240 ml/day of pomegranate juice for 90 days did not significantly change blood pressure in another study [134]. Probably because Aviram *et al.* (2001) used in-house produced pomegranate juice unlike the other group that used a commercial juice with no information about anthocyanins content [135]. Furthermore, a supplementation using Medox™ (purified anthocyanins) capsules, corresponding to 320 mg/day anthocyanins for 12 weeks, decreased systolic blood pressure by 5% [136]. Additionally, a reduction in both systolic and diastolic blood pressure was observed with after chronic intakes (more than 500 mg/day anthocyanins) of freeze-dried blueberries and mixed berries [137], [138].

In contrast, several other trials reported no significant changes in blood pressure after anthocyanin consumption. In very recent studies, blueberries [139], bilberries [140], anthocyanin-rich orange juice [141], chokeberry juice [142], cranberry juice [143] and aronia extract [144] did not affect blood pressure. This might be because office blood pressure was measured in most of these studies rather than 24 h ambulatory blood pressure (the gold standard technique for blood pressure measurement) [116].

1.5.4.2. Effect of anthocyanins consumption on endothelial function

Endothelial dysfunction - especially the reduction in bioavailability of endothelium-derived nitric oxide (eNO) - is established as one of the important early events in atherosclerosis [145], [146]. Lack of bioavailable eNO leads to arterial stiffness and an increase in vasoconstriction and thereby increased blood pressure [147]. Measurement of flow-mediated vasodilation (FMD) and arterial stiffness reflect the activity of NO and the endothelial function [148], [149].

In several human interventions, the consumption of anthocyanins was linked to increased FMD and decreased arterial stiffness. In a very recent study, Curtis *et al.* (2019) reported that 1 cup per day of blueberries juice (corresponding to 364 mg anthocyanins) for 6 months improved endothelial function. FMD increased by 1.45%,

while arterial stiffness decreased by 2.24% in 138 obese and overweight volunteers [139]. Moreover, Istas *et al.* (2019) observed an increase in FMD after consumption of either aronia whole fruit or aronia extract for 12 weeks compared to the baseline of day 1 and the placebo [94]. Acute improvement in FMD was also observed. About 1.4% and 1.7% increase in FMD after 2h consumption of aronia extract compared to baseline and placebo, respectively. A correlation between anthocyanins metabolites found in plasma after aronia consumption, especially isoferulic, dihydroferulic and hydroxybenzoic acids, was suggested as the cause of this improvement. However, no significant changes in arterial stiffness were observed in the same study [94]. Another study published this year found an increase in FMD by 2.3% following 2 and 6 h after the consumption of 150 mg anthocyanins derived from Medox™ or blueberry extract. The improvement was also dose dependant and correlated with phenolic metabolites present in circulation after the intervention. No changes in arterial stiffness was found [131]. Furthermore, in hypertensive postmenopausal women, freeze-dried blueberry containing 470 mg of anthocyanins for 2 months decreased brachial-ankle pulse wave velocity (1,401±122 cm/second; $p<0.01$; baseline levels were 1,498±179 cm/second) which was similar to another study which examined the effect of six week consumption of dehydrated powder of blueberry on arterial stiffness [150], [151].

On the other hand, many other studies reported non-significant changes after anthocyanin-rich fruit and vegetable consumption [152]–[155]. For instance, Hollands *et al.* (2018) found that anthocyanin-rich orange juice did not affect the carotid-femoral pulse wave velocity and brachial-ankle pulse wave velocity which are measurements reflect the arterial stiffness nor affect NO concentration in plasma [141].

1.5.4.3. Effect of anthocyanins consumption on lipid profile.

Elevated levels of lipid - mainly LDL - are the key risk factor for initiation and development of atherosclerosis [156], [157]. On the other hand, HDL has a protective role by promoting reverse cholesterol transport; the process by which cholesterol is transported to liver for excretion and thereby reducing the progress of atherosclerosis [158]–[160]. Thus, the effects of anthocyanin consumption on lipid profiles have received much attention [161]–[163]. Several human interventions observed an improvement in lipid profile after the intake of fruit and vegetables and anthocyanin-rich extracts. Wallace *et al.* (2016) systematically reviewed many randomized controlled trials reporting the effect of anthocyanins on CVD biomarkers [164]. Of twelve eligible assessed articles, ten studies investigated the effect of anthocyanins

on lipid profile. Nine of the ten studies included LDL as an outcome. Four of these nine articles reported a significant decrease in LDL [162], [165]–[167]. The reduction varied from 10.72 to 22.9 %. Interestingly, only the trials conducted in participants with initially elevated cholesterol levels showed a significant decrease in LDL. Moreover, total cholesterol was significantly reduced in three studies [162], [166], [168]. Triglyceride significantly decreased only in two reviewed articles. Those two studies were conducted in hyperlipidemia subjects [162], [166]. On the other hand, six articles reported a significant increase in HDL circulating levels [62], [162], [168], [169]. Three of them were conducted with hyperlipidemia subjects and the increase varied from 3.6 to 34 %. It seems that the people with hypercholesterolemia or with elevated risk biomarkers were influenced more by anthocyanin since the significant improvements were reported mainly in trials conducted with dyslipidemics.

In addition to the trials that were reviewed by Wallace *et al.* (2016), an additional ten trials were recently published (Table 1.3). Two of three articles that showed a significant decrease in cholesterol and LDL were conducted in obese patients or former smokers. As shown in Table 1.3, bilberries caused the highest improvement in lipid profile with an increase in HDL by 22% and reduction in total cholesterol, LDL and triglyceride by 8.5, 16.2 and 17.8%, respectively. However, the trial was conducted without a control group. The population in this study was composed of healthy subjects [140].

Most trials used a whole food material or (poly)phenolic-rich extracts that might have contained several bioactives alongside anthocyanins. To the best of my knowledge, only seven trials have been reported to use a mixture of purified anthocyanins extracted from bilberry and blackcurrant called Medox™ [136], [165], [167], [170]–[173]. None of them reported a significant decrease in total cholesterol, while LDL significantly decreased in four reports [136], [165], [167], [170]. The participants in all these four reports were hypercholesterolemic supporting the notion that the people with cholesterol levels higher than normal are the most influenced by anthocyanin treatment.

Table 1. 3: Overview of effects of dietary anthocyanins on lipid profile in randomized controlled trials

Material	Doses (mg/day)	Duration	Participants	Cholesterol	LDL	HDL	TAG	Ref
Red orange juice ‡	250	12 weeks	11 obese women	-7.1 (ns)	-10.8 *	-4.5 (ns)	+2.9 (ns)	[132]
Medox™	160	16 weeks	27 cognitive impairment	Ns	ns	ns	ns	[173]
Blueberry juice	364	6 months	115 obese	+3.5 (ns)	+1.5 (ns)	+5*	+5.6 (ns)	[139]
	182			0	-1.5 (ns)	+1.6 (ns)	+15.9*	
Bilberries ‡	~200 §	6 weeks	11 healthy men	-16.2*	-8.5*	+22.1*	-17.8*	[140]
			25 healthy women	-10.8*	-13.7*	+11.8*	-36.5*	
Red orange ≠	50	28 days	41 healthy	0	-3.0 (ns)	+7.4 (ns)	-7.7 (ns)	[141]

- Values are % of change compared to control group. * = significant changes; (ns) = not significant; ‡ = no control groups; ≠ = the control group was blond orange; ND = not determined; ¥ = the change in blood lipid levels after intervention were not mentioned in the text; § = dose was estimated based on the given dose of 150 g three times a week of bilberries (3038 mg/kg material)
-

Continued

Material	Doses (mg/day)	Duration	Participants	Cholesterol	LDL	HDL	TAG	Ref
Aronia extract	30	12 weeks	66 healthy	-4.6 (ns)	-4 (ns)	0	-10(ns)	[94]
Aronia whole fruit	3.3			-7.5 (ns)	-9.1 (ns)	-7.7 (ns)	0	
Chokeberry juice	113.3	4 weeks	84 healthy	-1.2 (ns)	-0.3 (ns)	ND	+1.6	[142]
	28.3			-1.4 (ns)	+0.3 (ns)	ND	-2.2	
Wild blueberry drink ¥		28 days	40 healthy	(ns)	(ns)	(ns)	(ns)	[131]
Medox™	320	28 days	16 healthy	-5.9 (ns)	0	-4.8 (ns)	-10.2 9ns)	[172]
Aronia extract	45.1	12 weeks	49 healthy, former smokers	-8*	-11*	+3.2 (ns)	+3.5 (ns)	[144]

- Values are % of change compared to control group. * = significant changes; (ns) = not significant; ‡ = no control groups; ≠ = the control group was blond orange; ND = not determined; ¥ = the change in blood lipid levels after intervention were not mentioned in the text; § = dose was estimated based on the given dose of 150 g three times a week of bilberries (3038 mg/kg material)

Although the randomized control trials showed that anthocyanins have the potential to reduce the risk of CVD and atherosclerosis, there was inconsistency within studies due to the limitations in many of these trials. The main reasons for this inconsistency could be that many of these trials were conducted without a proper control group [42], [174]–[181]; the doses used hugely varied within studies from 0.5 to 512 mg/day [42], [182], and the duration varied from two weeks to one year [64], [183]; the health status of subjects were varied within studies, and most importantly, whole materials that contain several bioactives were used in most of these trials instead of using purified anthocyanins which make the effectiveness of anthocyanins on CVD biomarkers is inconclusive. Thus, further investigations using suitable controls and purified anthocyanins are still required.

1.5.5. Atherosclerosis and HDL function

HDL is well recognized as having an important role in protecting against atherosclerosis [184]. Many epidemiological and clinical studies confirmed that high levels of HDL in circulation is inversely associated with the incidence of CVD, mainly CHD [185]–[188]. However, several recent studies suggest that the anti-atheroprotective effect of HDL is more complex [189]. Studies that were conducted in participants with some mutations that cause decreases in HDL concentration showed enhanced anti-atherogenicity [190], [191].

Moreover, patients given an inhibitor of cholesteryl-ester transfer protein (CETP) enzyme – an enzyme which facilitates the transport of cholesterol ester between lipoproteins - showed a higher risk of CHD although the substantial increase in HDL levels suggesting that the anti-atheroprotective effects of HDL is beyond the concentration and its more related with HDL properties and functions [192]. Therefore, HDL function is gaining more attention and it is becoming more accepted that HDL quality is more important than quantity [193].

HDL has many anti-inflammatory, anti-atherogenic and anti-oxidative functions [194]–[196]. One of the most established functions is cholesterol efflux from foam cells, the hall mark of atherosclerosis, through reverse cholesterol transport pathway [197]–[199]. The more cholesterol transferred by HDL, the slower atherosclerotic plaque size can grow. In fact, apolipoprotein A1 (Apo A1), the major apolipoprotein presents in HDL, plays a central role in this pathway. The lipid-poor Apo A1 increases the affinity of HDL to bind with cholesterol transporters in macrophages such as the ATP-binding cassettes A1 (ABCA1) and the ATP-binding cassettes G1 (ABCG1) and then

rapidly acquire phospholipid and unesterified cholesterol from peripheral tissues, thereby facilitating cholesterol efflux. The overexpression of ApoA1 promotes RCT and maturation of HDL [200]–[203]. For more details about the role of HDL in RCT, see sections 2.2. HDL-associated enzymes such as CETP protein and lecithin-cholesterol acyl transferase (LCAT) play essential roles in the net transfer of cholesterol from foam cells to HDL, and in modulating HDL shape [204], [205].

The second key function of HDL is antioxidation activity. HDL can protect LDL from oxidation and thereby attenuate the progress of atherosclerosis. HDL-associated antioxidant enzymes such as paraoxonase 1 (PON1) seem to be the main mediators of this property. PON1 can protect LDL from oxidation as well as hydrolysing the ox-LDL. It was also found that high levels of PON1 increased the cholesterol efflux capacity of HDL. In addition to PON1, other enzymes were found to contribute to antioxidation such as acetyl-hydrolase platelet activation factor [206]–[208]. For more details about the role of PON1 as biomarker of HDL function in protecting LDL from oxidation, see section 3.2.

Another important property of HDL related to its anti-atherogenicity is the heterogeneity of HDL particles. HDL has two subclasses; HDL2 and HDL3; based on the density of the HDL particle. HDL2 is larger and less dense than HDL3 [197]. HDL3 was found to have a strong inverse association with CHD [209]. Furthermore, 2-dimensional electrophoresis revealed at least 12 subspecies of HDL. Pre-β-1 and α-3 particle were strongly associated with CHD, while, α-1, pre-α-3 and pre-α-1 were lower in CHD patients [210].

On the other hand, dysfunctional HDL may exert pro-atherosclerotic and pro-inflammatory functions. An increase in ApoA-associated enzyme myeloperoxidase decrease the cholesterol efflux capacity of HDL. Additionally, serum amyloid A clusterin was higher in dysfunctional HDL [211]–[213]. Fig 1.4 summarize the key important properties of anti-atherogenic and dysfunctional HDL.

Several human interventions have reported an improvement in HDL biomarkers after consumption of anthocyanins. For instance, Zhu *et al.* (2014) reported an increase in cholesterol efflux capacity and PON1 activity after consumption of purified anthocyanins. Similarly, Qin *et al.* (2019) found that anthocyanins increased cholesterol efflux capacity and decreased serum CETP mass and activity [165], [170]. In addition, blueberry anthocyanins increased Apo A1 levels and the density of HDL particles [139].

Although, the importance of HDL function in attenuation of atherosclerosis is apparent, little research has investigated the effect of anthocyanins consumption on the key elements of HDL function, and the mechanism by which anthocyanins might improve HDL function has yet to be studied; and more investigation is still required.

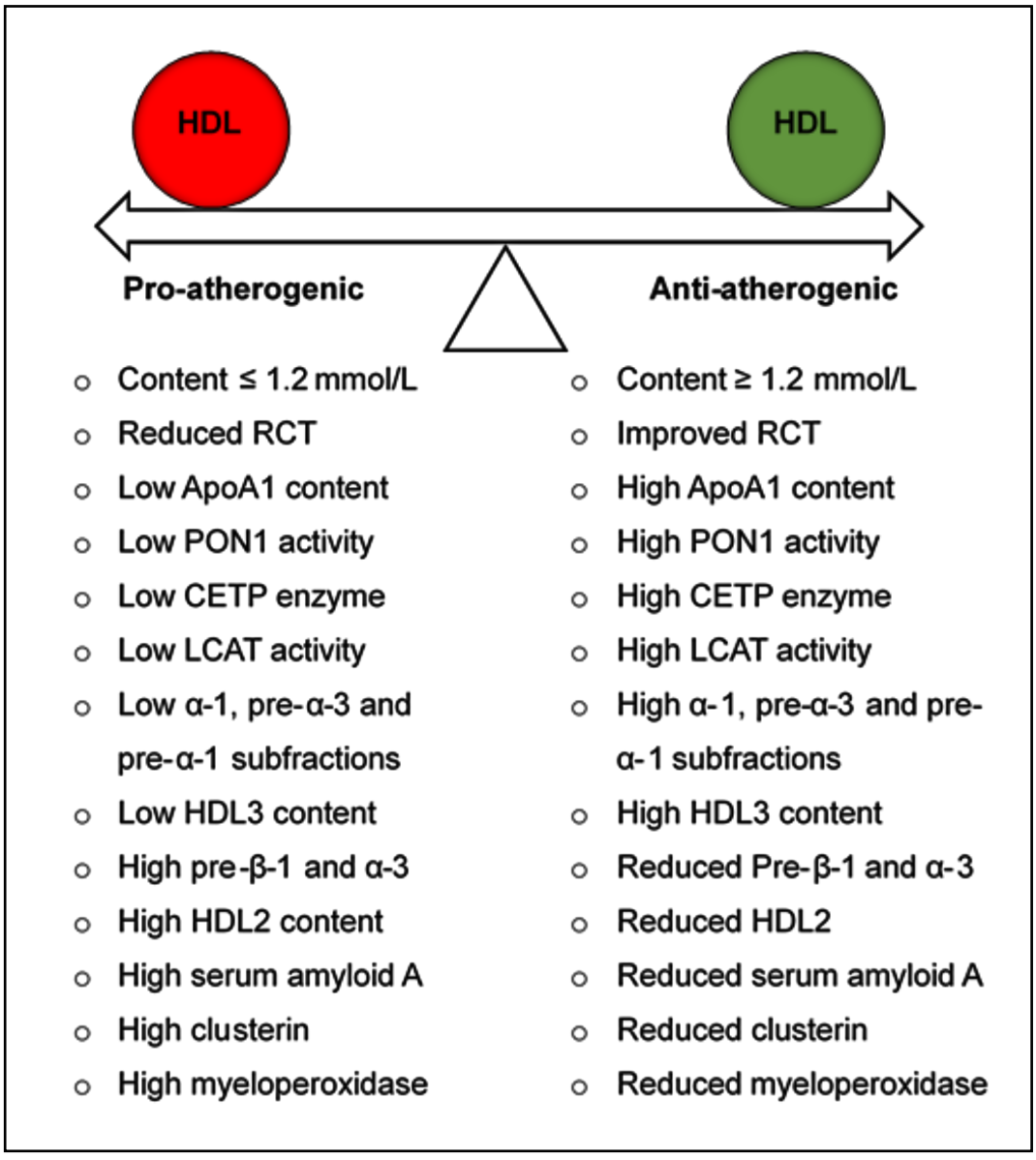


Figure 1. 4: Scheme demonstrate the key elements of antiatherogenic vs proatherogenic HDL.

ApoA1= apolipoprotein A1, PON1= paraoxonase 1, CETP= cholesteryl-ester transfer protein, LCAT= lecithin-cholesterol acyl transferase.

1.6. Thesis aims

Considering the previous literature summarized in this chapter, there are several questions that need to be investigated. Firstly, the mechanisms of action by which anthocyanins would exhibit their effect still have not been elucidated and more research is still required. Secondly, with all limitations of previous clinical trials, it is hard to draw general conclusions regarding the effectiveness of anthocyanins and therefore, conducting rigorous trials with purified anthocyanins is essential. In addition, anthocyanins are rapidly degraded producing a wide range of metabolites suggesting that the metabolites may be more beneficial, but the biological activities of most of them have not been investigated or were investigated individually ignoring the possibility of interaction. The tested concentrations were often higher than the physiologically achievable one, thus, more research still requited to fill this gap. Since high lipid levels are the main CVD risk that drives the development of atherosclerosis, this project focused on the effect of anthocyanins on lipid/lipoprotein profile and quality and the pathways related to that. To address these questions and progress the research from previous current literature, the overall aims and objectives are outlined below.

- **Overall aim** of this project is to develop a mechanistic understanding of how anthocyanins alter lipid/lipoprotein metabolism.

- **The overall hypothesis** is that anthocyanins and/or their metabolites, will affect lipid/lipoprotein metabolism and/or quality by altering the expression of key genes involved in reverse cholesterol transport (RCT) and/or the quality of circulating lipoproteins.

RCT was proposed to be the target of anthocyanin treatment. There are different elements involved in RCT. However, the cholesterol transporters, scavenger receptors and HDL function are the key ones. Therefore, these three elements were the main focus of the project. To test the overall aim and hypothesis, the following objectives were addressed:

1- To study the effects of anthocyanins and their metabolites on the expression of key genes involved in RCT

Two important elements in RCT were studied in this objective; the effects of the parent anthocyanins and their metabolites on cholesterol transporters and scavenger receptors, using physiologically-relevant concentrations. The upregulation of

cholesterol transporters and the downregulation of scavenger receptors should result in a promotion in RCT and a reduction in cholesterol accumulation in the foam cell. Chapter two of this thesis aims to address this question using in-vitro and in-vivo models.

2- To determine the effects of anthocyanins and their metabolites on PON1 gene expression and PON1 enzyme activities as a marker of HDL function

HDL function is important to promote RCT. HDL carries the cholesterol back to the liver for excretion and therefore, improvement in HDL function should result in an increase in its capacity to carry more cholesterol from foam cells and attenuate the progress of atherosclerosis. PON1 contribute in many functions of HDL. Thereby, an increase in PON1 gene expression or activities may enhance the functions of HDL. Due to the little research that has been carried out to understand this effect, the main objective for chapter three was to address this question using an in-vitro models.

3- To detect any atherosclerosis-linked genes and pathways affected by the anthocyanins using RNA sequencing.

It is important to understand the mechanism(s) by which anthocyanins deliver their beneficial effects. Because, anthocyanins may target several pathways, non-targeted approaches such as RNA sequencing would be useful to elucidate the possible underlying molecular mechanism(s) for further in-vitro and in-vivo investigation. In-vitro cell model was used to address this objective which was presented in chapter four.

4- To compare the effects of 4-week consumption of cyanidin-type anthocyanins and delphinidin-type anthocyanins on lipid/lipoprotein profiles and markers of HDL function using a randomized placebo-controlled cross-over human trial.

This objective was addressed in chapter five by conducting a randomized cross-over placebo-controlled trial. The trial was designed to compare the effects of two types of anthocyanins on lipid profile/HDL function in a population with elevated cholesterol levels using two purified anthocyanin-rich extracts. The trial was also conducted to translate the in-vitro findings in previous chapters to an in-vivo situation and to gain insight into the interaction between anthocyanins treatment, PON1 genotype, PON1 activities and markers of HDL function.

CHAPTER TWO

Chapter two: The effects of dietary anthocyanins and their metabolites on the expression of key genes and the function of reverse cholesterol transport (RCT)

2.1. Abstract

Data from various epidemiological studies, human interventions and animal interventions studies indicate that dietary anthocyanins consumption has the potential to lower the risk of cardiovascular disease (CVD) and modulate cholesterol metabolism, however, the mechanisms remain undeciphered. It has also been shown that anthocyanins are rapidly degraded, and it is most likely that its metabolites are responsible for high bioactivity. Upregulating reverse cholesterol transport (RCT) was proposed as a mechanism by which anthocyanins exhibit their effects on cholesterol level. Therefore, the aim of the research described in this chapter was to investigate the ability of anthocyanins and their metabolites to enhance the expression of ABCA1 and ABCG1 genes, and downregulate the expression of scavenger receptor genes which are key genes involved in RCT. Two approaches have been used, *in vitro* and *in vivo*. For *in vitro*, human and mouse macrophages were treated with two predominant types of anthocyanins and 18 of their recently identified metabolites at physiologically relevant concentrations. In addition, the effects of 8 pure synthetic phase II conjugates of anthocyanin metabolites on RCT key genes were investigated. In vivo effects were investigated by quantifying gene expression in the liver and aorta tissues isolated from *Apo E*$^{-/-}$ mice that had been fed for 16 weeks with anthocyanin- and flavonol-rich tomatoes, compared to mice consuming anthocyanin-free red tomatoes and anthocyanin-free but flavonol-rich tomatoes. The expression of RCT key genes was quantified using qPCR. None of the anthocyanins or their metabolites affected the expression of RCT key genes in macrophages. Furthermore, there were not any synergistic effects as a mixture of anthocyanins and various of their metabolites also did not cause changes in gene expression. The in vitro model was validated using two positive controls which stimulated ABCA1/G1 expression, showing that the model was working properly. Moreover, these findings were consistent with the lack of effect of the anthocyanin-supplemented mouse diets on the expression of these genes in liver and aortic tissue, compared to red tomato. There was a small increase in ABCG1 gene expression in aortic tissue of the anthocyanins-rich tomato fed group compared to the control group, but this was not statistically significant. These data do not support the notion that anthocyanins and their metabolites affect the gene expression of cholesterol transporters and receptors in macrophages and mouse tissue.

2.2. Introduction

Numerous reported animal studies, epidemiological surveys and clinical observations have revealed a positive relationship between LDL and total cholesterol levels in blood and CVD incidence [159]. Any excess cholesterol can accumulate in peripheral tissues and lead to pathological consequences as cholesterol cannot be catabolized in organs and tissues, only in the liver and steroidogenic tissues which can convert cholesterol to bile acids or steroid hormones. Therefore, cells need to maintain cholesterol homeostasis and regulate utilization of intra-and extracellular cholesterol sources [214]. Many cells do that by regulating the activities of the hydroxymethylglutaryl-CoA (HMG-Co-A) synthase and reductase enzymes, and regulating LDL-receptor synthesis, thereby preventing any further intracellular cholesterol synthesis or extracellular LDL-cholesterol entry [215].

In contrast, macrophages (phagocytes which can take up dead cells, modified lipoproteins, and other extracellular debris such as aggregated lipoproteins) can accumulate a large amount of cholesterol. Because of its toxicity, free cholesterol (FC) is usually esterified to cholesteryl esters (CE) which can be stored as cytosolic lipid droplets. As macrophages progressively scavenge more and more cholesterol, they take the appearance of foam cells because of the foamy appearance of cytoplasmic cholesteryl esters inclusions. The accumulation of cholesterol-laden macrophages (foam cells) in the intima of arteries is the early hallmark of atherosclerosis [156]

As such, the limitation of cholesterol accumulation to reduce the atherosclerosis progression in macrophages is necessary and the only way to do this is to efflux or eliminate the accumulated cholesterol by a process called reverse cholesterol transport (RCT) [156] **.**

2.2.1. Reverse Cholesterol Transport

Reverse cholesterol transport (RCT) is the process by which excess cholesterol is effluxed from peripheral tissues and is delivered to HDL and then transported to the liver for elimination and excretion as bile acids**.** Cholesterol-laden macrophages (foam cells) are the main cell type which participates in plaque formation and usually RCT is studied as a macrophage-specific phenomenon in relation to atherosclerosis**.** Active cholesterol efflux is the main way by which macrophages efflux cholesterol to the extracellular acceptor. ATP-binding cassette (ABC) transporters are the key players in this mechanism [158], [216]**.**

2.2.2. ATP-Binding Cassette transporters and RCT.

ATP-Binding Cassette transporter type A1 (ABCA1) and type G1 (ABCG1), belong to the large ABC transporter family and are the main regulators of RCT in macrophages. These transporters bind with ATP to provide the required energy for active transportation. The role of ABCA1 in RCT was not known until it was reported that ABCA1 knockout mice show similar symptoms as patients suffering from Tangier disease – low HDL levels in plasma, low ApoA1 levels and high accumulated CE, which are related to the deficiency in cholesterol transport to Apo A1 [214].

2.2.3. RCT mode of action

Fig 2.1 summarizes the main steps of the RCT process and the role of ABCA1 and ABCG1 transporters. The main cause of foam cell generation is the internalization of modified LDL-cholesterol. This triggers the enzyme machinery of macrophages such as acyl coenzymeA:cholesterol acyltransferase-1 (ACAT1) which aids in the esterification of cholesterol and produce cholesterol esters (CE) for storage [217], [218]. When CE becomes highly accumulated in macrophages, cholesteryl ester hydrolase and lysosomal acid lipase convert CE to free cholesterol. ABCA1 and ABCG1 only transport free cholesterol [218]. Excess of free cholesterol leads to production of oxysterol (an oxidized derivative of cholesterol). Oxysterol is a liver X receptor (LXR) ligand which is required to upregulate ABCA1 and ABCG1 synthesis[214].

As a result of ABCA1 synthesis, a direct interaction occurs between ABCA1 and lipid-poor or lipid-free apolipoprotein A1 (mainly produced by the liver). This binding triggers a multi-step process by which free cholesterol (unesterified) and phospholipids are transferred to Apo A-1 to produce a nascent HDL particle. After that, lecithin:cholesterol acyl transferase (LCAT) present in HDL particles triggers what is called HDL maturation by esterifying the cholesterol that has been transported to HDL. This results in the production of globular HDL particles with a normal CE core, thereby, reducing free cholesterol at the surface of HDL and increasing further the capacity of HDL to accept additional cholesterol [214], [219]. Subsequently, ABCG1 acts to transport cholesterol from inside to the surface of the macrophage where it is accepted by the HDL particle, thus completing cholesterol elimination. ABCG1 transports additional cholesterol to HDL producing what are called mature HDL particles [220]. The mature HDL then travels to the liver or steroidogenic tissues where a selective uptake of HDL-cholesterol can occur mediated by scavenger receptor type B1 (SR-B1) [214].

Once cholesterol is returned to the liver, cholesterol can be used as a precursor for bile acids production in a process mainly mediated by the cytochrome P450 enzyme, cholesterol 7 α-hydroxylase (CYP7A1). Bile acids subsequently are secreted into bile duct and may be excreted via the faeces or retaken up from the intestine via a process called enterohepatic recirculation [221].

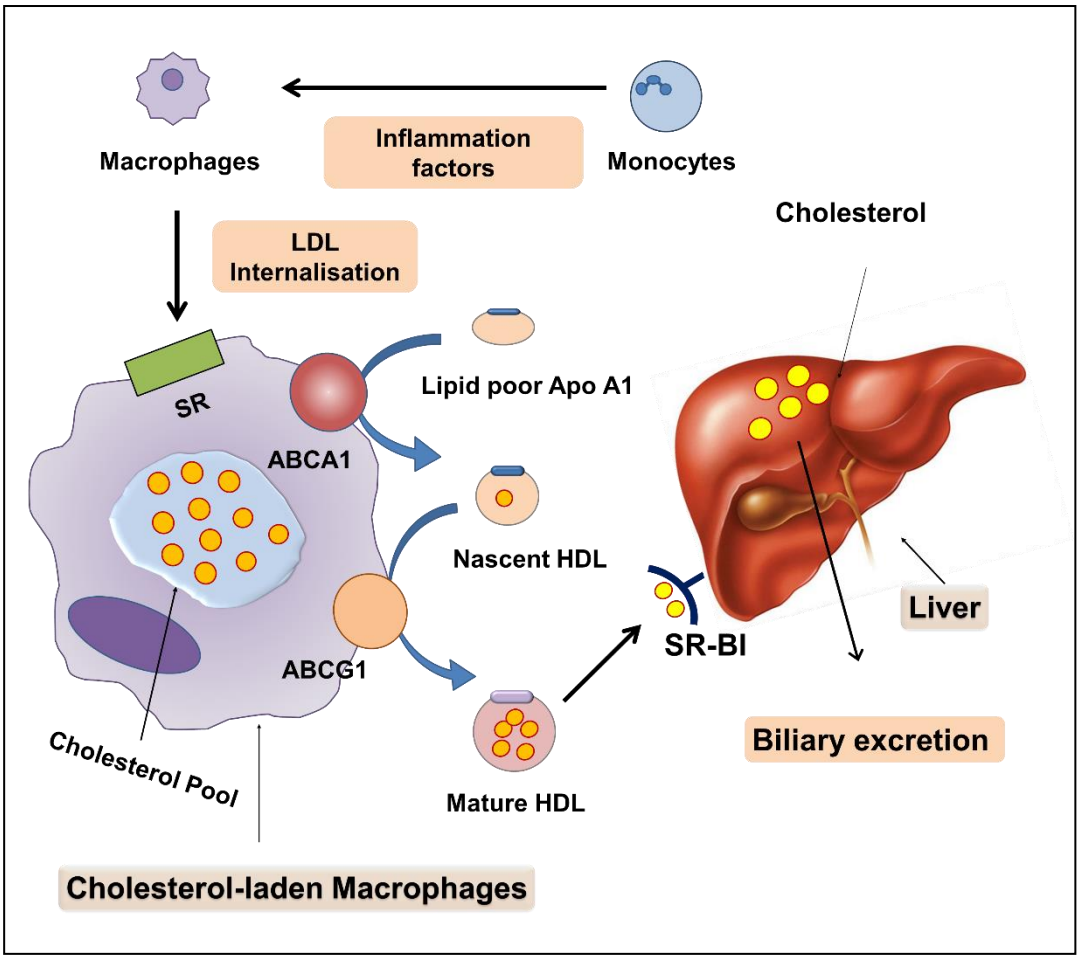


Figure 2. 1: A model depicting the principal process of reverse cholesterol transport in macrophages and the role of ABCA1 and ABCG1.

To prevent the progress of foam cell formation, cholesterol laden macrophages transfer cholesterol via ABCA1 to lipid-poor apo A-I forming nascent HDL particles which are subjected to further lipidation via ABCG1 to produce mature HDL particles. Mature HDL particles deliver cholesterol to the liver by SR class B type I (SR-BI) for bile acid production.

2.2.4. Scavenger receptors

Scavenger receptors (SR) play a significant role in atherosclerosis [222]. While native LDL can enter into cells via LDL receptors which can be negatively regulated by cholesterol stores, modified LDL such as ox-LDL or acetylated-LDL can only bind with scavenger receptors leading to foam cell formation [223]. Scavenger receptors can bind with different ligands and therefore they can be classified into several subfamilies. However, the class A and class B are the principal receptors for binding modified LDL in macrophages.

SR-A1 (belonging to class A) and CD36 (belonging to class B) account for 75 to 90% of the uptake of acetylated and ox-LDL [224]. The downregulation of SR-A1 and CD36 in *Apo E*$^{-/-}$ mice reduced foam cell formation by reducing modified LDL uptake [225].

2.2.5. Effect of anthocyanins on cholesterol efflux and ABCA1/G1 gene expression.

A few recent reports have described the effect of anthocyanins and their metabolites on cholesterol efflux and ABCA1/G1 gene expression. Wang, Y (2012) investigated the effects of the anthocyanin C3G in apolipoprotein E-deficient mice (*Apo E*$^{-/-}$) and reported that C3G (2 g/kg diet) upregulated ABCG1 protein expression in the aortic tissue of the mice [226]. *In vitro*, Wu (2014) investigated the effect of some anthocyanin metabolites such as ferulic acid, gallic acid and vanillic acid at relatively low concentrations on cholesterol efflux in RAW264.7 mouse macrophages and reported that ferulic acid and gallic acid increased cholesterol efflux from the mouse macrophages at 10 µM by 1.4 and 1.3 fold, respectively, whereas, vanillic acid had no effect on cholesterol efflux [227]. Wang, D. (2012) studied the effect of C3G and PCA on cholesterol efflux and ABCA1/G1 gene expression in mouse and human-derived macrophages at concentrations very close to that found *in vivo* and reported that C3G had no effect on cholesterol efflux at concentrations between 0.25 to 1 µM, while PCA significantly increased the cholesterol efflux at the same concentrations in both mouse and human-derived macrophages. Additionally, it was reported that PCA but not C3Gat concentrations between 0.25 and 1 µM upregulated both ABCA1 and ABCG1 gene expression by 2.5 and 4-fold, respectively, in human-derived macrophages (THP-1 macrophages). It was also reported that PCA down-regulated the presence of the microRNA miRNA-10b, which is responsible for the repression of ABCA1/G1 gene expression [221]**.**

The results of these studies support the notion that anthocyanins achieve their biological action via their metabolites. However, these studies did not take into

account that anthocyanin metabolites are found in blood as a complex mixture and it is possible that synergistic interactions action may take place. In addition, there are no reports of the activities of metabolite phase-2 conjugates. Therefore, additional studies using mixtures of anthocyanin metabolites and metabolite conjugates are still needed.

2.3. Objectives

The overall aim of the experiments described in this chapter was to investigate the impact of anthocyanins and their metabolites on processes involved in reverse cholesterol transport. The specific objectives for this chapter were to (1) investigate the ability of anthocyanins and their metabolites to upregulate the expression of cholesterol transporters genes; (2) to investigate the ability of anthocyanins and their metabolites to downregulate the expression of scavenger receptors and (3) to investigate the influence of feeding anthocyanin-expressing purple tomatoes in comparison to anthocyanin-free red tomatoes on the expression of cholesterol transporters and scavenger receptors in aorta and liver using the *Apo* $E^{-/-}$ mouse model.

2.4. Materials and methods

2.4.1. Cell cultures

THP-1 human monocytes were obtained from the European Collection of Authenticate Cell Cultures (ECACC). The cells were routinely cultured as suspended cell in bicarbonate-buffered RPMI-1640 media containing 2 mM glutamine (Sigma-Aldrich, Cat. # R8758), 10% Fetal Bovine Serum (FBS) (v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Thermofisher, Cat# 15140122) according to the manufacture's protocol and incubated at 37°C in 5% CO_2. Once the cells had grown to confluence (0.8 x 10^6 cell/ml), they were split or seeded for differentiation. THP-1 monocytes were chemically differentiated to macrophages by phorbol 12-myristate 13-acetate (PMA, from Sigma-Aldrich, Cat. # P8139). For differentiation, THP-1 monocytes were seeded at 5 x 10^5 cell/ml in 12-well plates and treated with 100 ng/ml PMA for 48 hr followed by a 24-hr rest time in culture media without PMA (Fig 2.2). This differentiation process was monitored by following the morphological transition of the monocytes from suspended to adherent cells. All experiments were conducted between passages 9 to 15.

J774A.1 mouse macrophages was obtained from European Collection of Authenticate Cell Cultures (ECACC) and routinely cultured in the same conditions as mentioned with THP-1. For seeding, the cells were harvested at 80% confluency using a cell scraper and centrifugation for 5 min at 200 X g and seeded in 12-well plates. The cells then were left to attach to the plate for 48 hr. Because J774A.1 cells are already macrophages, the differentiation step was not required.

The differentiated THP-1 cells and J774A.1 macrophages were then preloaded with 25 µg/ml acetylated -LDL (ac-LDL) (Alfa Aesar, Cat. # J65029) for 24 hr prior the treatments (Fig 2.2). The non-preloaded cells were treated with full growth media free from ac-LDL. All procedures were performed according to Wang *et al.* 2012 [228]

2.4.2. Treatments

The major human metabolites arising from cyanidin-3-glucoside and the predicted metabolites of delphinidin-3-glucoside were assessed at concentrations of 1 and/or 10 µM for 24 hr (Fig 2.2). Standard solutions were initially prepared in 100% DMSO at concentrations of 10 mM followed by dilution to the final concentrations using FBS-free cell culture media, immediately prior to application to cells. Final treatment concentration of DMSO was 0.1%. The vehicle control was preloaded cells treated with DMSO at concentration of 0.1%. Kaempferol (10 µg/ml, 30 µM) and cyclic-

adenosine monophosphate (c-AMP) (300 µM) served as positive control (+ve), while, the negative control (-ve) was non-preloaded cells treated with DMSO. Treatments were conducted in triplicate and the experiments were repeated two times.

C3G and D3G were purchased from Extrasynthese. PGA, PCA, gallic acid, ferulic acid, vanillic acid, 4HBA, sinapic acid, hippuric acid, 5HFA, c-AMP and kaempferol were purchased from Sigma Aldrich. Syringic acid purchased from Alfa Aesar and phloroglucinol was purchased from Across Organics. PCA conjugates and gallic acid conjugates were synthesised inhouse using the method by [229].

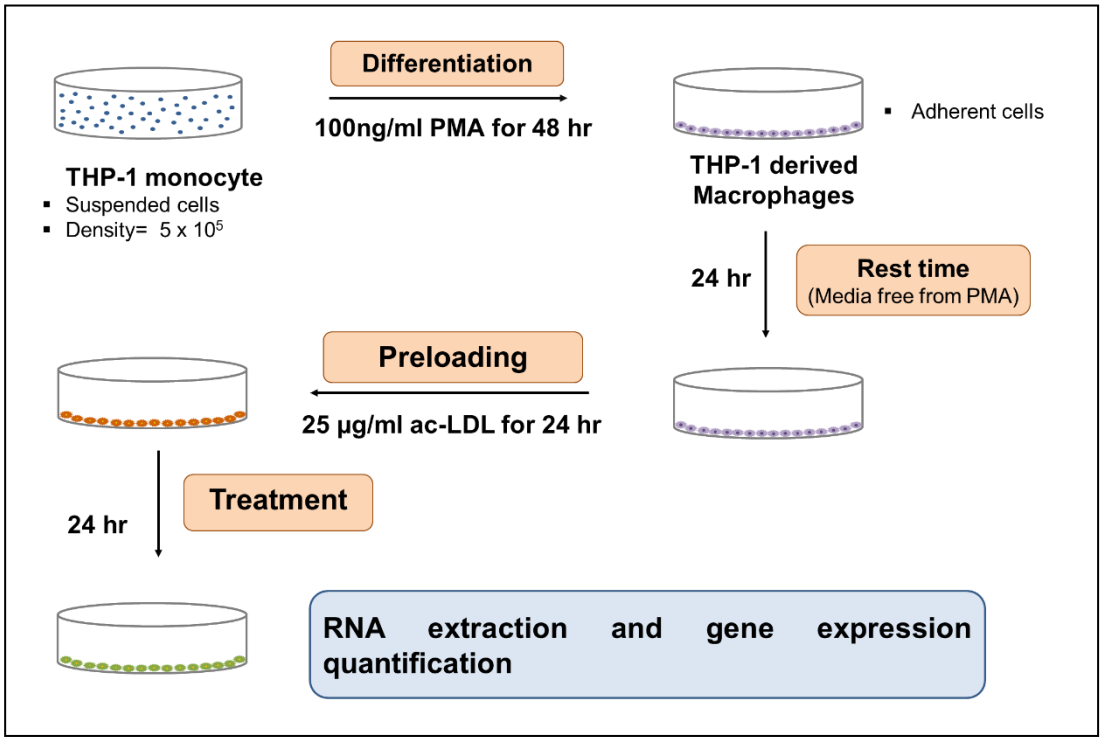


Figure 2. 2: Graphic demonstration of THP-1 differentiation, preloading and treatment.

PMA = phorbol 12-myristate 13-acetate. Ac-LDL = acetylated- DL

2.4.3. Selection of anthocyanins and their metabolites

There are many types of anthocyanins that produce a large number of metabolites. C3G alone produced 35 metabolites in serum, urine and faeces which makes it difficult to test all anthocyanins metabolites [73]. Therefore, it was essential to establish a list of metabolites of interest that will be examined based on their presence in serum and their concentrations. Cyanidin and delphinidin are the most common anthocyanidins found in human diet, thus, C3G, D3G and their metabolites were the main focus of my project [17].

It was well established that C3G degrades into phloroglucinaldehyde (PGA) and protocatechuic acid (PCA) following the deglycosylation of C3G and releasing the aglycone (cyanidin) at physiological pH and temperature. PGA is derived from the A-ring, while, PCA is derived from the B-ring [230][41] . Therefore, the effects of cyanidin, PGA and PCA were investigated.

Additionally, about 17 metabolites have been identified in serum when pure ^{13}C-labelled C3G was fed to humans. Although, PCA was not the major metabolite in this study, 13 derivatives of PCA were detected with hippuric acid, vanillic acid, ferulic acid and 4-hydroxybenzaldehyde (4HBA) representing the predominant metabolites in serum [73]. Therefore, the effects of vanillic acid, ferulic acid, 4-hydroxybenzaldehyde were studied. Moreover, C3G and its metabolites undergo a subsequent conjugation via phase II metabolisms in liver producing wide range of glucuronides, sulphates and methyl forms. Phase II conjugates of PCA including PCA glucuronides , PCA sulphates and methylated PCA (such as vanillic acid) reached to 2.35 μM in serum after feeding with isotope-labelled C3G to human [75]. Therefore, PCA-3-glucuronide (PCA-3GlcA), PCA-4-glucuronide (PCA-4-GlcA), PCA-3-sulphate (PCA-3-Sulph) and PCA-4-sulphate (PCA-4-Sulph) were included to the list of metabolites of interest. Fig 2.3 demonstrates the possible pathways of C3G and PCA biotransformation.

Since ferulic is one of the most abundant metabolites of C3G present in serum, it was considered that ferulic acid was released directly from C3G as caffeic acid that was subsequently methylated to ferulic acid rather than a derivatization of PCA (Fig 2.3). If this pathway was taken into account, phloroglucinol would be released as the A-ring product. So far no one has reported phloroglucinol as an anthocyanin metabolite.

However, in our lab we could identify phloroglucinol in the urine of humans who had consumed purified anthocyanin-rich extracts (data unpublished) supporting the notion that C3G may degrade into ferulic acid and phloroglucinol (Fig 2.3). Phloroglucinol, therefore, was added to the list of compounds of interest.

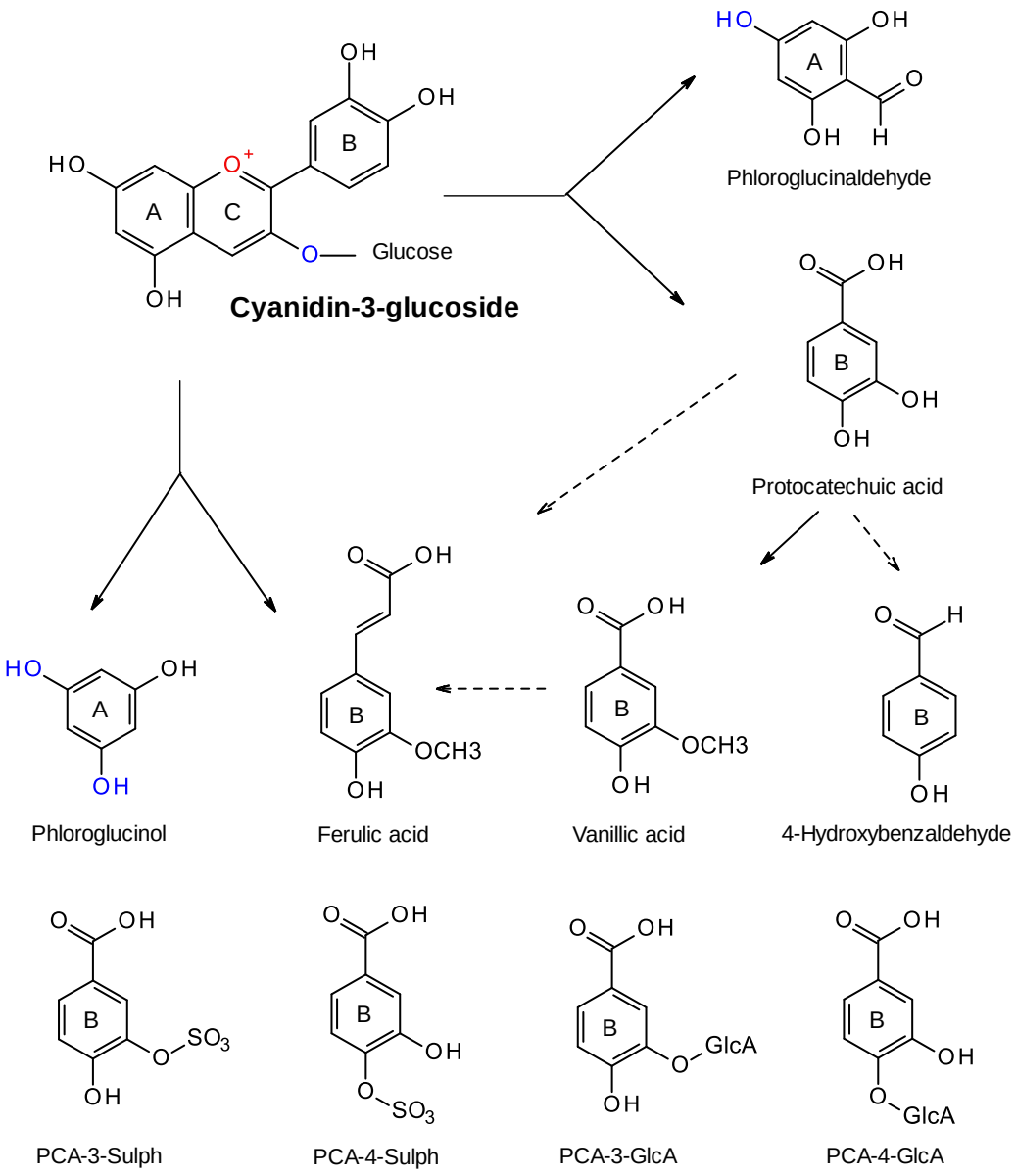


Figure 2. 3: Structures and proposed pathway for C3G metabolism.

PCA-3-sulph= PCA-3-sulphate, PCA-4-Sulph= PCA-4-sulphate, PCA-4-GlcA= PCA-3-glucuronide, PCA-4-GlcA= PCA-4-glucuronide. AccelrysDraw (version 4.2 for windows) was used to draw the chemical structures.

On the other hand, no human intervention feeding solely pure D3G to participants has been reported. However, there is evidence from studies feeding D3G-rich fruit extracts that supports the idea that D3G metabolism follows a similar pattern as that of C3G degradation. D3G degrades rapidly to gallic acid and PGA in culture media (pH=7.4, temperature 37°C) [231]. Gallic acid originates from the B-ring of delphinidin (Fig 2.4). Gallic acid per se can undergo phase II metabolism to produce 3-*O*-methylgallic acid (3MeGA) and 4-O-methylgallic acid (4MeGA) [232] and possibly gallic acid-3-glucuronide (GA-3-GlcA) and gallic acid-4-glucuronide (GA-4GlcA). Therefore, the effect of gallic acid and gallic acid conjugates were also tested.

In addition, syringic acid was found to be the major degradant in human plasma after consumption of anthocyanin-rich bilberry extract which contains mainly trihydroxylated-B ring anthocyanins such as D3G [91]. It has been suggested that syringic acid originated from methylation of gallic acid [91][233] (Fig 2.4). Thus, the effect of syringic acid was also tested. In this study, vanillic acid, HBA, PGA, gallic acid and hydroxybenzoic acid were also reported as bilberry's anthocyanin metabolites.

Similar to C3G, phloroglucinol might be generated from the A-ring of D3G, thus, sinapic acid would be one of the B-ring metabolites of D3G. Moreover, sinapic acid might produce 5-hydroxy ferulic acid (5HFA) after undergoing demethylation, the process that can occur easily in presence of microbiota (Fig 2.3). Although, I am not aware of any previous studies reporting sinapic acid and 5HFA as anthocyanin metabolites, they might be present in circulation for a short time and cause an effect before fast derivatization into other compounds. Therefore, these two metabolites were also added to the list.

The concentrations of metabolites detected human plasma after consumption of ^{13}C-labelled C3G ranged from 0.1 - 2 μM, with cumulative concentrations of metabolites reaching 10 μM. Therefore, the selected metabolites were tested at 1 and 10 μM as representative of low and high physiological concentrations.

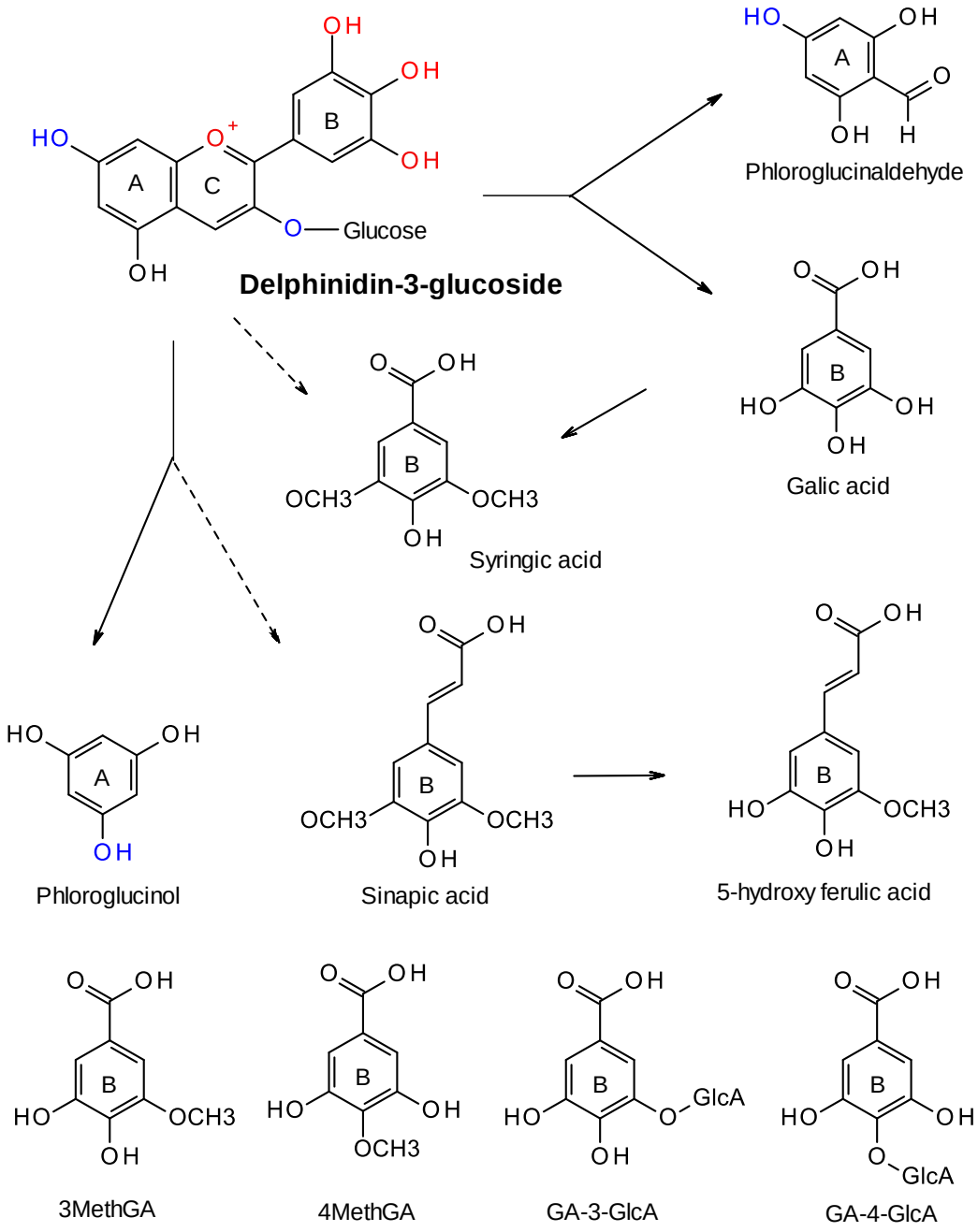


Figure 2. 4: Structures and proposed pathway for D3G metabolism.

3MethGA= 3-*O*-methylgallic acid, 4MethGA= 4-*O*-methylgallic acid, GA-4-GlcA= gallic-3-glucuronide, GA-4-GlcA= gallic acid-4-glucuronide. AccelrysDraw (version 4.2 for windows) was used to draw the chemical structures.

Based on the justifications provided above, 20 compounds of anthocyanins and their metabolites were chosen and tested at two different physiologically relevant concentrations (1 and 10 μM). These compounds are: C3G, D3G, PCA, PGA, ferulic acid, vanillic acid, 4HBA, gallic acid, syringic acid, sinapic acid, 5HFA, PCA-3-GlcA, PCA-4-GlcA, PCA-3-Sulph, PCA-4-Sulph, GA-3-GlcA, GA-4-GlcA, 3MethGA and 4MethGA. Since there was not a human study that used solely pure D3G, it was difficult to confirm whether syringic acid, sinapic acid, 5HFA and phloroglucinol were from D3G or from other bilberry's anthocyanins. Therefore, these metabolites have been tested as predicted (potential) metabolites at a concentration of 10 μM.

There are many other metabolites that might be important and need to be examined such as vanillic acid sulphate that was detected at considerable amounts (430 nM) following ^{13}C-labelled C3G consumption [73]. However, it was not available in our lab. Only PCA and gallic acid conjugates were available in our lab when the experiment was conducted and therefore, the project focused on them. In addition, there are new metabolites that discovered recently while writing this thesis after conducting the experiment and there was not enough time to examine them. For instance, in a very recent study, phenylacetic acid and 3-(4-hydroxyphenyl) propionic acid were detected following the consumption of aronia crude extract. These compounds and others should be investigated in future work [94].

2.4.4. RNA extraction

RNA was extracted from treated THP-1-derived macrophages and J774A.1 mouse macrophage using RNeasy® Mini Kit (Qiagen Ltd, Cat# 74106) according to the manufacturer's protocol. Briefly, cell culture media was removed, and cells were washed once with cold PBS (Thermofisher, Cat# 14190094). Cells layer were then harvested using a cell scraper and centrifuged for 1 min at 1000 x g. The pellets were initially lysed using the provided RLT Buffer and further homogenised using a QIAshredder (Qiagen Ltd, Cat. # 79654,). The quantity of RNA, as well as 260/280 and 260/230 ratios were measured using the NanoDrop ND-1000 spectrophotometer. Three readings for RNA quantification were obtained per sample and the average RNA concentration was then calculated. RNA samples were aliquoted and stored at -80°C until use.

2.4.5. Animal study

The study was approved by the Animal Welfare and Ethical Review Body (AWERB) at the University of East Anglia and conducted in compliance with the provisions of

the Animals (Scientific Procedures) Act 1986 (ASPA) and the LASA Guiding Principles for Preparing and Undertaking Aseptic Surgery.

The experiment protocol and procedures were described by Day *et al.* 2018 [234]. Briefly, 92 male homozygous ApoE-deficient mice were purchased from Jackson Laboratories (Charles River Laboratories, Kent, United Kingdom) and were kept in acclimatisation on standard breeding diet AIN-93G (Testdiets, Kent, United Kingdom). At age 8 weeks, the mice were then randomly divided into five groups and fed ad libitum supplemented with 10% tomato powder. The tomatoes were genetically modified to express one type of polyphenol. The five group of mice were fed with 10% tomato powder that express either flavonols (n=20), a mixture of anthocyanins and flavonols (n=20), resveratrol (n=20), isoflavones (n=12) or control low- or no-polyphenol red tomato powder (control, n=20) for 17 weeks. The body weight and food consumption of the mice were measured three times a week and mice were sacrificed by exsanguination under isoflurane anaesthesia. The organs were washed with 0.9% saline and EDTA by direct injection in the heart's left ventricle. Aortic and hepatic tissue for gene expression was then collected immediately and stored in RNAlater (Life Technologies, Paisley, UK) overnight and stored at -20 °C until further processing. The study was conducted by Mark Winterbone at QIB and my role was to process the tissues, extract RNA and do the assays.

2.4.6. RNA extraction from aorta and liver

Aortas were placed in RNAlater to remove the surrounding adipose tissue using SZ40 Olympus Zoom dissecting microscope. Two to three aortas were then pooled for total RNA extraction to obtain enough RNA for gene expression. Total RNA was extracted using miRNeasy Mini Kits (Qiagen Ltd, Cat. # 217004) according to the manufacturer's instructions. The tissue was lysed using QIAzol Lysis Reagent (Qiagen Ltd, Cat. # 79306). The homogenization step was done by using Precellys 24 lysis & homogeniser at 6000 rpm for 4 cycles for 30 s (Bertin Technologies, France) and QIAshredder.

Livers were processed into a homogeneous powder using a pestle and mortar under liquid nitrogen. Total RNA was extracted using miRNeasy Mini Kits according to the manufacturer's protocol. The lysis step was performed by adding 700 µl QIAzol Lysis Reagent to 25 mg homogenized sample followed by a homogenisation step similarly to aortas tissues. RNA quantity, 260/230 ratio and 260/280 ratio were measured as previously mentioned.

2.4.7. Gene expression quantification

For the quantification of gene expression of ABCA1 and ABCG1 in THP-1 and in mouse cells and tissues, Precision OneStepPLUS qRT-PCR master mix (Primerdesign, Cat. # OneStepPLUS and OneStepPLUS-LR) and the pre-designed probe/primers for the genes ABCA1, ABCG1, GAPDH, 18S, YWHAZ and RPL4 (from IDT technology) were used. The sequences for forward and reverse primers and probes are given in Table 2.1 and 2.2. The preparation of the samples and reaction mixes for the RT-PCR was done using the CAS-1200 robot and Robotics 4 software (Corbett Life Sciences) in 96 well Semi-Skirted fast plates with final volume 20 µl contains 20 ng total RNA and appropriate concentrations of primers and probes. RNAse-free water (Qiagen Ltd, Cat. # 129112) was used as a no template control (NTC). Each sample was run as three technical replicates.

RT-qPCR was performed using ABI StepOne Plus machine (Applied Biosystems) with conditions of 48°C for 30 min for reverse transcription followed by one cycle at 95°C for10 min and then 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. In this technique, RNA can be used as starting material and within one step the complimentary DNA is produced, and the target gene amplified. A fluorescent signal is generated by the use of dual labelled probes and Taq polymerase.

For scavenger receptors gene expression in (MSR1, SCARB1 and CD36), reverse transcription was carried out to produce c-DNA using 1 µg of total RNA and the Precision NanoScript Reverse Transcription kit (Primerdesign, Cat. # RT-NanoScript2) in a final volume of 20 µl according to the manufacturer's instructions. Appropriate concentrations of primers were used. RNAse-free water (Qiagen Ltd, Cat. # 129112) was used as a No Template control (NTC). Each sample was run as three technical replicates. primers were designed according to Day *et al.* 2018 [234] using either the Roche probe library software https://lifescience.roche.com/en_gb/brands/universal-probe-library.html or the PubMed primer designing tool https://www.ncbi.nlm.nih.gov/tools/primer-blast/ and checked for specificity using BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi Table 2.1 and 2.2. Gene expression analysis was carried out according to Day *et al.* 2018 using the VIIA™ 7 PCR System (Life Technologies, UK) in a final reaction volume of 10 µl, and comprised of 1 X ImmoMix PCR MasterMix (Bioline, Cat. # BIO-25022), SYBR Green (0.06ul of 100x stock), ROX reference dye (175 nM) magnesium (0.5 mM), BSA (50 µg/ml) and 10 nM forward and reverse primers [234]. The following PCR cycling conditions were used; one cycle at 95°C for 10

min for initial denaturation, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min and then the melt curve. For each gene, the melt curves and standard curves were performed to determine the primer specificity and linearity respectively.

Target genes were normalized against housekeeping genes. (1) GAPDH for THP-1, (2) 18S for J774A.1, (3) YWHAZ for liver and RLP4 for aorta. The fold expression of the target genes was quantified relative to the control sample (DMSO for cell model or red tomato for mice tissues) using $2^{-\Delta\Delta Ct}$ method.

2.4.8. Statistical analysis

All data and statistics were carried out by using GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). All values are given as means ± SD. Any statistical difference between the groups was determined with one-way ANOVA coupled with Dunnett's Multiple Comparison Test comparing all sample groups to control (DMSO in cell model or red tomato in mouse tissues). Values of $p \leq 0.05$ were considered significant.

Table 2. 1: Primer and probe sequences of genes analysed in THP-1 human macrophages

Gene name		Sequence
ABCA1	Forward	AGA TAA TCC CCT GAA CCC AAG
	Reverse	GCT ACC CAC CCT ATG AAC AAC
	Probe	5`-/56-FAM/TTC CAA ATA/ZEN/AAG CCA TGC CCT CTG C/3IABkFQ/-3`
ABCG1	Forward	TGG TCT GAG TCA CAC ATG C
	Reverse	GTC TGA ACT GCC CAA CCT AC
	probe	5`-/56-FAM/TGC AAC CTC /ZEN/CAT GAC AAA ATC TGC TG/3IABkFQ/-3
GAPDH	Forward	TGT AGT TGA GGT CAA TGA AGG G
	Reverse	ACA TCG CTC AGA CAC CAT G
	probe	5`-/56-FAM/AAG GTC GGA/ZEN/GTC AAC GGA TTT GGT C/3IABkFQ/-3
MSR1	Forward	TTT GAT GCT CGC TCA ATG AC
	Reverse	TTG AAG GGA AGG GCT GTT TT
SCARB1	Forward	TAT GCC CAG TAC GTC CTC CT
	Reverse	CTG GCT CAC GGT GTC CTC
CD36	Forward	TGG AAC AGA GGC TGA CAA CTT
	Reverse	TTG ATT TTG ATA GAT ATG GGA TGC

Table 2. 2: Primer and probe sequences of genes analysed in mice tissue and J774A.1 mouse macrophages.

Gene name		Sequence
ABCA1	Forward	CCA TAC CGA AAC TCG TTC ACC
	Reverse	CCG CAG ACA TCC TTC AGA ATC
	probe	5`-/56-FAM/CGT ACG TGC/ZEN/AGA TCA TAG CAA AGA GCT/3IABkFQ/-3
ABCG1	Forward	ACT GTT CTG ATC CCC GTA CT
	Reverse	TCT CCA ATC TCG TGC CGT A
	probe	5`-/56-FAM/TGC CAC TTC/ZEN/CAT GAC AAA GTC TGC T/3IABkFQ/-3
RPL4	Forward	CTT GCC AGC TCT CAT TCT CTG
	Reverse	TGG TGG TTG AAG ATA AGG TTG A
	probe	5`-/56-FAM/CTG AAC AGC/ZEN/CTC CTT GGT CTT CTT GTA/3IABkFQ/-3
YWHAZ	Forward	AGC TTG GCC TTC TGC AC
	Reverse	AGA TCA GGG ACA GAG TCT CAG
	probe	5`-/56-FAM/ACC CAC TCC/ZEN/GGA CAC AGA ATA TCC A/3IABkFQ/-3
18S	Forward	CTT TGG TCG CTC GCT CCT C
	Reverse	TCA CCG GGT TGG TTT TGA TC
	probe	5`-/56-FAM/TGC CGA CGG/ZEN/GCG CTG ACC /3IABkFQ/-3
MSR1	Forward	CTG GAC AAA CTG GTC CAC CT
	Reverse	GTC CCC GAT CAC CTT TAA CA
SCARB1	Forward	CGT TGT CAT GAT CCT CAT GGT
	Reverse	ACA GGC TGC TCG GGT CTA T
CD36	Forward	TTG AAA AGT CTC GGA CAT TGA G
	Reverse	TCA GAT CCG AAC ACA GCG TA

2.5. Results

2.5.1. The *in vitro* effect of anthocyanins and their metabolites on cholesterol transporters gene expression.

2.5.1.1. Effect of anthocyanins parent compounds on ABCA1 and ABCG1 genes expression in THP-1 macrophages

The effect of two anthocyanin parent compounds, C3G and D3G, on the expression of ABCA1 and ABCG1 genes was examined by incubating preloaded THP-1-macrophages with 1 and 10 μM of C3G and D3G for 24 hr (Fig 2.5 A and B). The cells were preloaded with 25 μg/ml ac-LDL for 24 hr prior to treatment to initiate foam cell formation and to stimulate the RCT process. The expression of ABCA1 and ABCG1 (the cholesterol transporters) was quantified using qPCR. As shown in Fig 2.5 A and B, there was no significant effect of C3G or D3G on ABCA1 and ABCG1. C3G at 10 μM slightly increased ABCG1 expression by 14% when compared to 1 μM, but it was not significant (Fig 2.5-B).

Kaempferol (the positive control) significantly induced the expression of the ABCA1 and ABCG1 genes by 2.7- and 4.2-fold, respectively ($p \leq 0.001$). On the other hand, the expression of ABCA1 and ABCG1 genes in the negative control (cells treated with DMSO but not preloaded with ac-LDL) was 40-50% lower than the ac-LDL pre-loaded control ($p \leq 0.001$, Fig 2.5 A&B). This indicates that the model is working and sensitive to treatment and that the ac-LDL preloading step does indeed increase the expression of the cholesterol efflux transporters ABCA1 and ABCG1 in THP-1 macrophages. Therefore, these data clearly show that treatment of the macrophages with anthocyanin parent compounds does not alter ABCA1 and ABCG1 gene expression. This was consistent with previous findings that C3G did not change ABCA1 and ABCG1 gene expression and indicated that the biological activity of anthocyanins may be attributed to their metabolites [228]. Therefore, the effect of anthocyanin metabolites on cholesterol transporters, ABCA1 and ABCG1, was investigated.

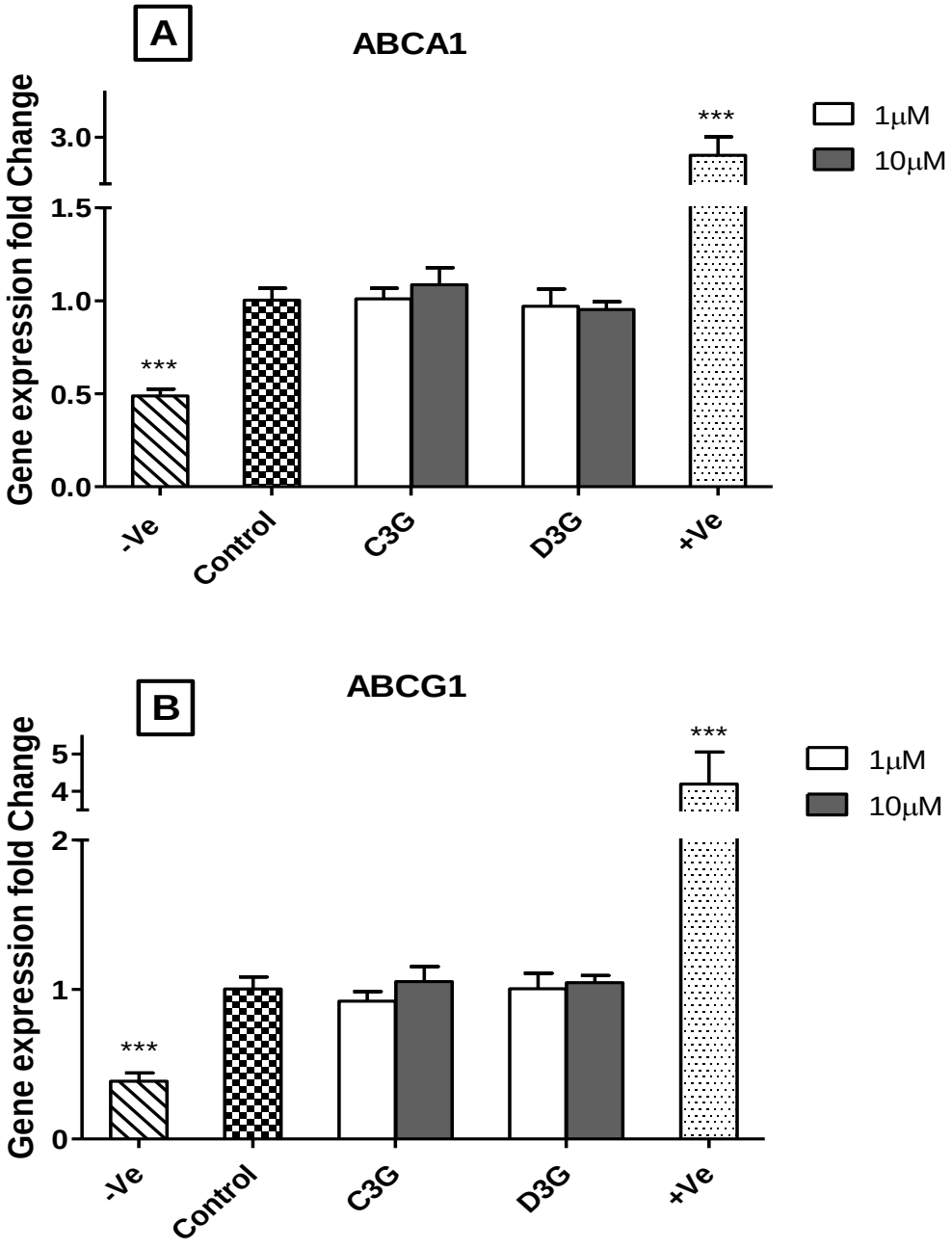


Figure 2. 5: Effect of anthocyanins parent compounds on (A) ABCA1 and (B) ABCG1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 μg/ml) and then treated; control = DMSO, C3G = cyanidin-3-glucoside, D3G = delphinidin-3-glucoside and +Ve = is the positive control, kaempferol (10 μg/ml, 30 μM). -Ve is the negative control which is cells treated with DMSO without preloading step. The fold change was calculated relative to the ac-LDL preloaded vehicle control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.5.1.2. Effects of anthocyanin metabolites on ABCA1 and ABCG1 gene expression in THP-1 macrophages.

Preloaded THP-1 macrophages were treated with 1 and 10 μM of PGA, PCA, gallic acid, ferulic acid, vanillic acid and 4HBA for 24 hr prior to RNA extraction and quantification of ABCA1 and ABCG1 gene expression (Fig 2.6 and 2.7). Similar to the parent compounds, there were no statistically significant differences observed at either of the concentrations tested on either ABCA1 or ABCG1 gene expression.

These data contradict with what has previously been reported, i.e. that 1 μM PCA upregulated both ABCA1 and ABCG1 gene expression in THP-1 macrophages by 2.6 and 4 fold, respectively [228]. Therefore, a series of experiments were carried out to confirm the sensitivity and reliability of the model and to validate my results. The data from these experiments has been presented later in this chapter.

Interestingly, ferulic acid (one of the main C3G metabolites) increased ABCA1 gene expression by 22% at the lower concentration tested which was similar to the concentration reported in serum (0.827 μM) after consumption of labelled-C3G [73]. However, this was not statistically significant (Fig 2.6). Unexpectedly, 4HBA at the higher but not the lower concentration reduced ABCG1 by 26% but again the change was not significant.

To further investigate the potential active compounds accounting for anthocyanin biological activity, syringic acid, sinapic acid, phloroglucinol and 5HFA were examined at concentration 10 μM (see Fig 2.8 A and B). However, no significant differences were found. Phloroglucinol, 5HFA and hippuric acid slightly decreased ABCG1 gene expression but the effect did not reach statistical significance (Fig 2.8 B).

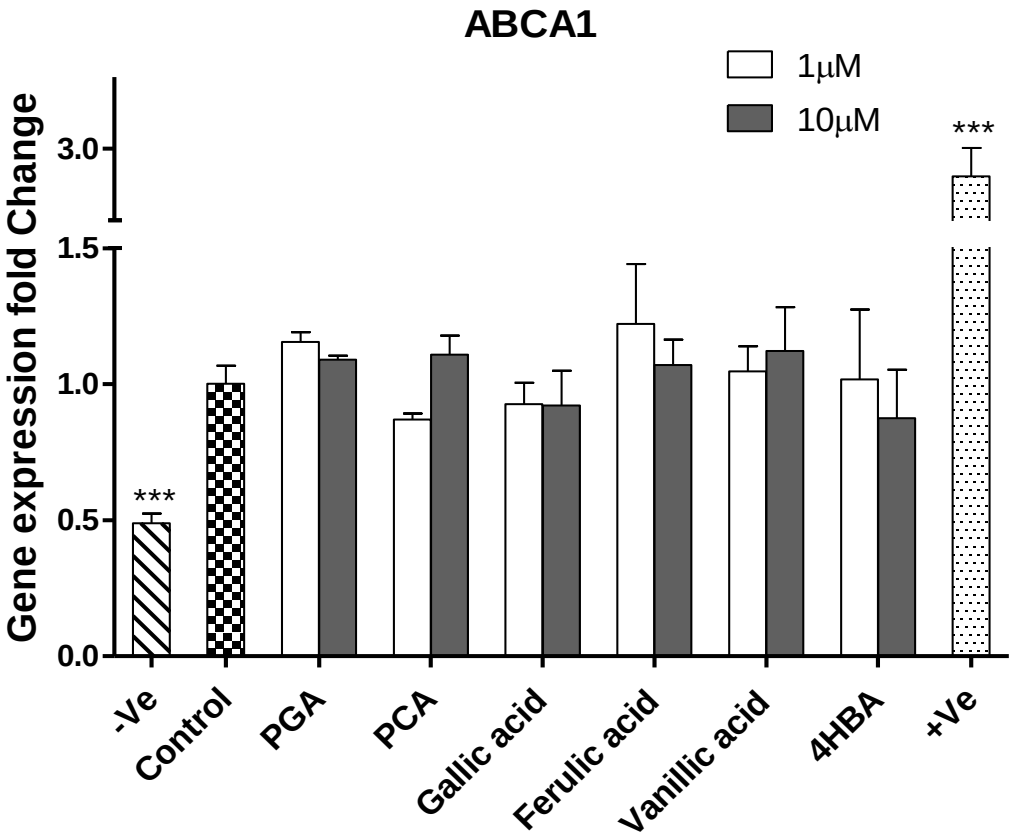


Figure 2. 6: Effect of anthocyanins metabolites on ABCA1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 μg/ml) for 24 hr prior treatment. Control = DMSO, PGA = phloroglucinaldehyde, PCA = protocatechuic acid, 4HBA = 4-hydroxybenzaldehyde and +Ve = the positive control, kaempferol (10 μg/ml, 30 μM). -Ve is the negative control which is cells treated with DMSO without preloading step. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

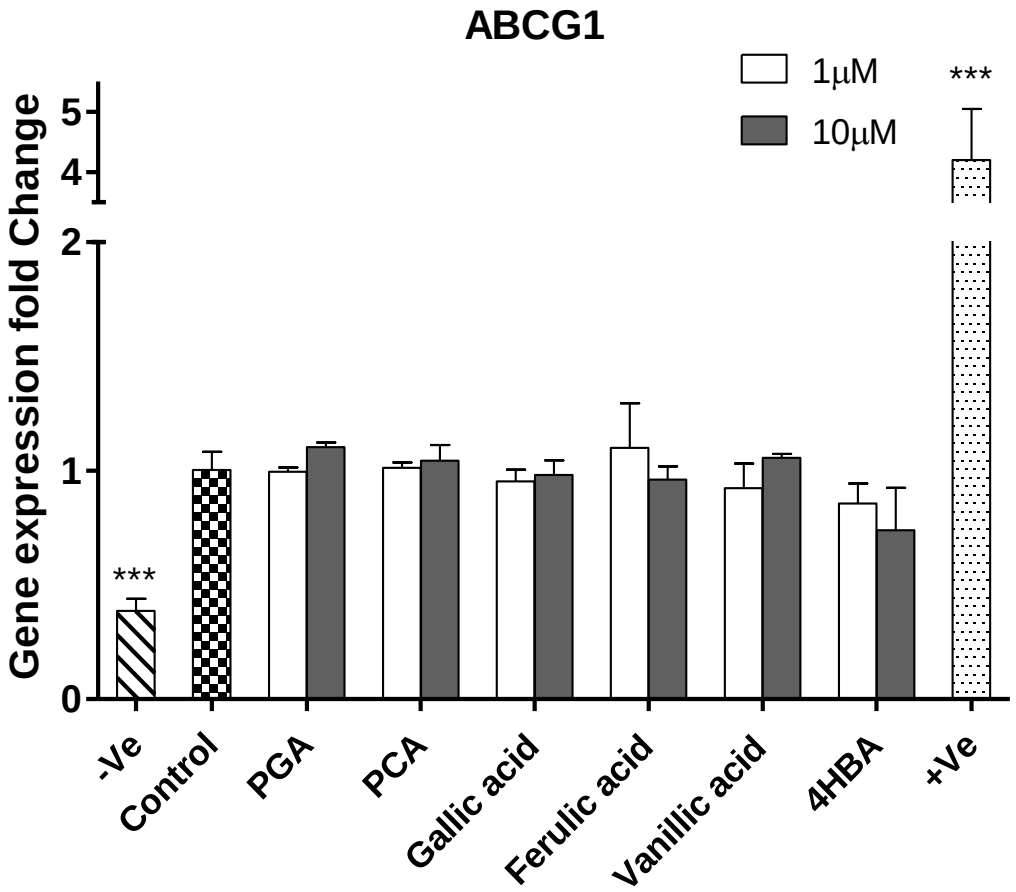


Figure 2. 7: Effect of anthocyanins metabolites on ABCG1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, PGA = phloroglucinaldehyde, PCA = protocatechuic acid, 4HBA = 4-hydroxybenzaldehyde and +Ve = the positive control, kaempferol (10 µg/ml, 30 µM). -Ve is the negative control which is cells treated with DMSO without preloading step. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

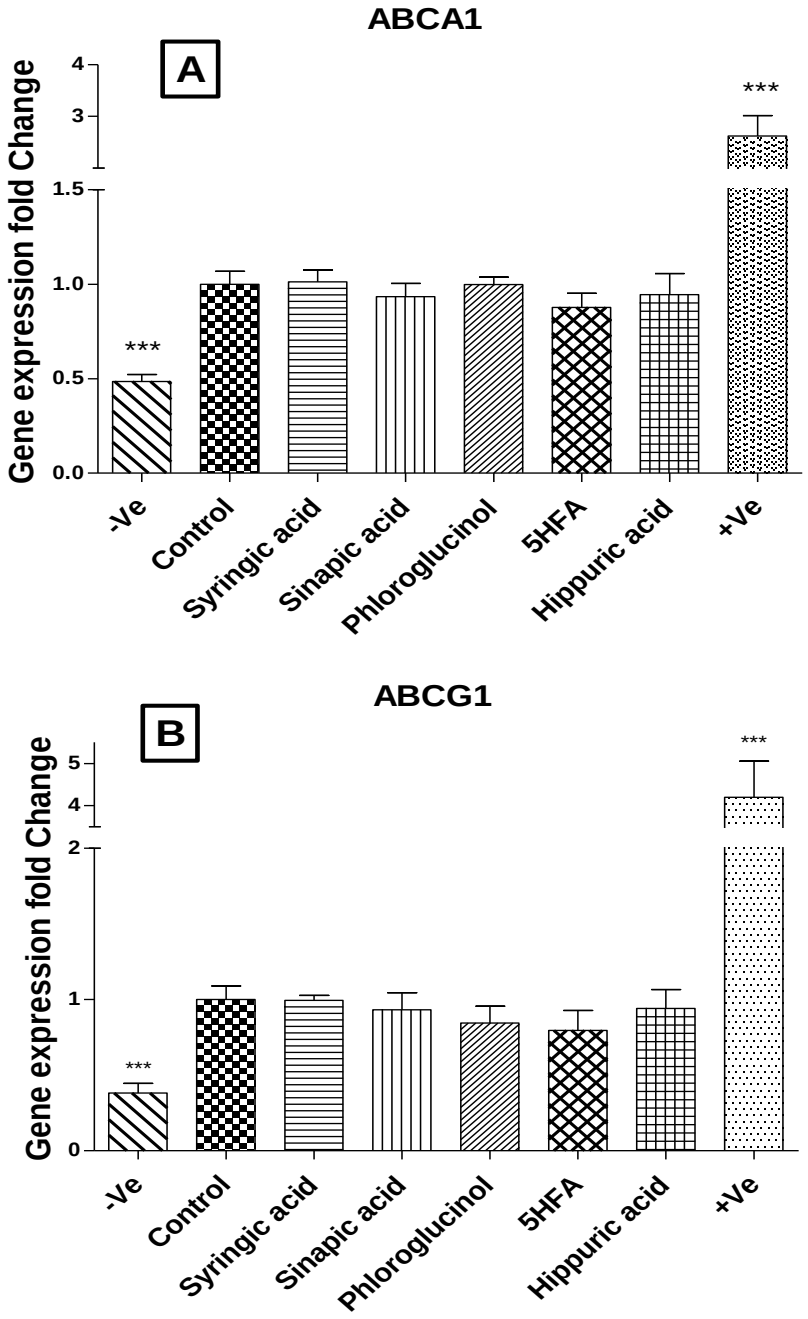


Figure 2. 8: Effect of anthocyanins potential metabolites on (A) ABCA1 and (B) ABCG1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, 5HFA = 5-hydroxyferulic acid and +Ve = the positive control, kaempferol (10 µg/ml, 30 µM). -Ve is the negative control which is cells treated with DMSO without preloading step. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.5.1.3. Effect of anthocyanin metabolite conjugates and mixtures on ABCA1 and ABCG1 gene expression in THP-1 macrophages.

PCA conjugates and gallic acid conjugates were investigated for their effect on the gene expression of the ABCA1 and ABCG1 cholesterol transporters using the same model as for the unconjugated metabolites of anthocyanins (Fig 2.9 and 2.10). To study the effect of metabolite conjugates, the cells were treated with a combination of PCA glucuronides (PCA-3-GlcA and PCA-4-GlcA, 1 µM each), and combination of PCA sulphates (PCA-3-Sulph and PCA-4-Sulph, 1 µM each) for 24 hr.

A mixture of C3G, PCA and PCA conjugates (1 µM each) was also incubated with cells to investigate the synergistic effect of C3G and PCA conjugates. Following the treatments, there was no significant changes on the gene expression of ABCA1 and ABCG1 with any of PCA conjugates (Fig 2.9). In addition, a mixture of C3G and PCA conjugates did not induce any changes in either of the cholesterol transporter genes (Fig 2.9).

To investigate the effect of gallic acid conjugates on ABCA1 and ABCG1 gene expression, the preloaded THP-1 macrophages were treated with a combination of gallic acid glucuronides (GA-3-GlcA and GA-4-GlcA, 1 µM each), a combination of methylated gallic acid (3MethGA and 4MethGA, 1 µM each) and a mixture of D3G, gallic acid and gallic acid conjugates, 1 µM each. Consistent with PCA conjugates, no significant change was seen with any of gallic acid conjugates or the mixture of D3G and gallic acid conjugates (Fig 2.10).

The data presented here show that none of the tested anthocyanin parent compounds or their metabolites caused significant changes in the expression of either the ABCA1 or ABCG1 genes. Apart from PCA, all of the data is novel in that there are no existing published reports describing the effects of any of these metabolites on the expression of RCT cholesterol transporter gene expression. However, since my data show that PCA did not induce the expression of ABCA1 or ABCG1 genes which contradicted a previously published report by Wang et al [228], additional experiments were conducted to examine the reliability and sensitivity of the model that was used in this current study.

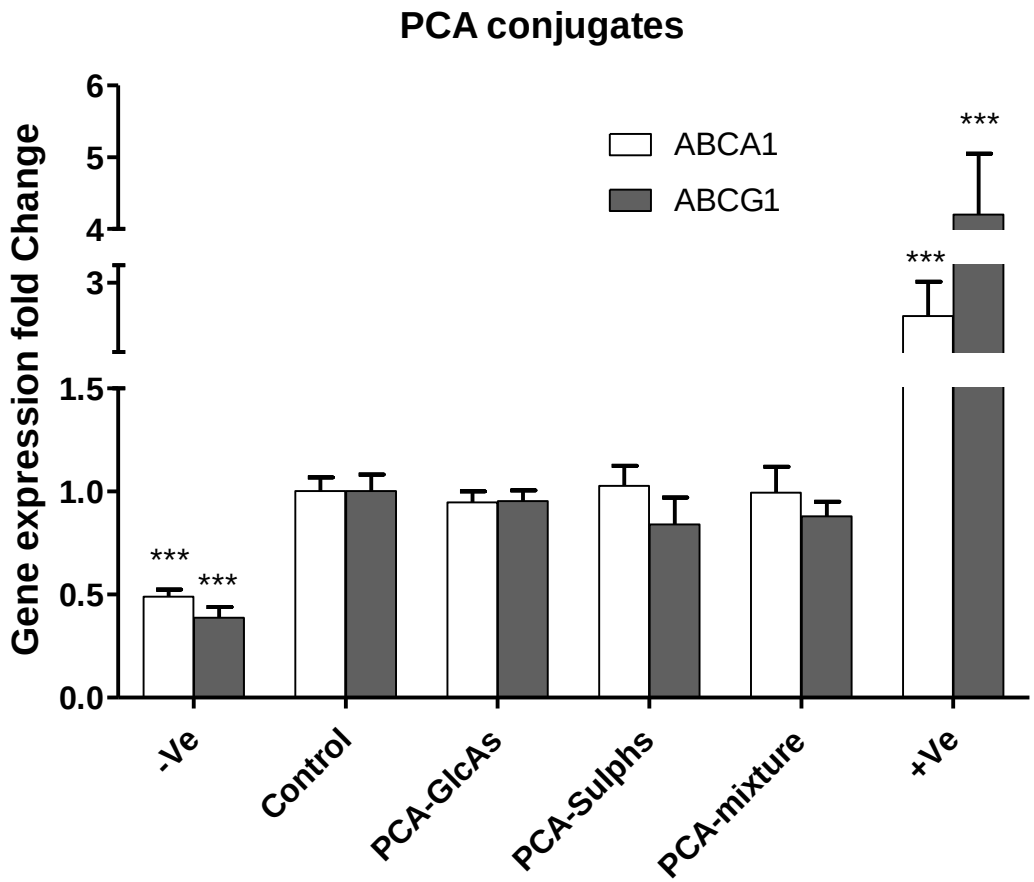


Figure 2. 9: Effect of PCA conjugates on ABCA1 and ABCG1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, PCA-GlcAs = 1 µM of PCA-3-glucuronide and PCA-4-glucuronide, PCA-Sulphs = 1 µM of PCA-3-sulphate and PCA-4-sulphate, PCA-Mixture = 1 µM of C3G, PCA and PCA conjugates. +Ve = is the positive control, kaempferol (10 µg/ml, 30 µM). -Ve is the negative control which is cells treated with DMSO without preloading step. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

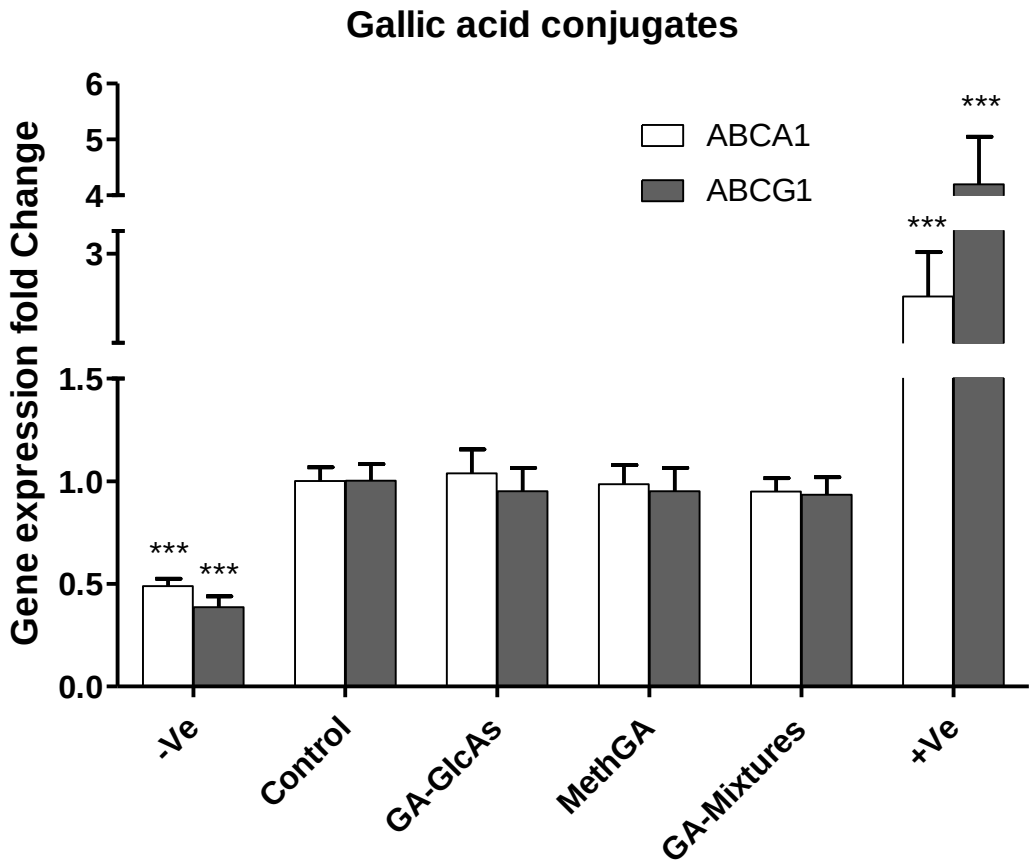


Figure 2. 10: Effect of gallic acid conjugates on ABCA1 and ABCG1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, GA-GlcAs = 1 µM of gallic acid-3-glucuronide and gallic acid-4-glucuronide, MethGA = 1 µM of 3-O- Methylgallic acid and 4-O- Methylgallic acid, GA-Mixture = 1 µM of D3G, gallic acid and gallic acid conjugates. +Ve = is the positive control, kaempferol (10 µg/ml, 30 µM). -Ve is the negative control which is cells treated with DMSO without preloading step. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.5.1.4. Repeatability and assay sensitivity

It has been reported that PCA upregulated both ABCA1 and ABCG1 gene expression by 2.6 and 4 fold, respectively, in preloaded THP-1 derived macrophages at a concentration of 1 μM [228]. It was also reported in the same paper that PCA treatment increased ABCA1 and to a lesser extent ABCG1 gene expression in ac-LDL-loaded mouse peritoneal macrophages (MPMs).

2.5.2.4.1. Using different cholesterol transporter inducer

Although kaempferol (the positive control) consistently increased both ABCA1 and ABCG1 gene expression in THP-1 at concentration 10 μg/ml in the current experiment, as expected, it is useful to examine whether the model is sensitive to any other RCT stimulators. Cyclic adenosine monophosphate (c-AMP) is a well-known ABCA1 inducer [235][236][237]. Therefore, the effects of c-AMP on ABCA1 gene expression was tested using the current model. Preloaded THP-1-derived macrophages were treated with c-AMP at a concentration of 300 μM and PCA at a concentration of 1 μM (Fig 2.11). c-AMP significantly upregulated ABCA1 gene expression by 1.8-fold, while, PCA did not significantly affect the gene expression (Fig 2.11, $p \leq 0.001$). This shows that the model is sensitive to treatment with at least two known agonists (kaempferol and c-AMP) but that expression of the ABCA1 and ABG1 genes is not altered by treatment with PCA.

2.5.2.4.2. The importance of the preloading step

In vivo, foam cell macrophages in arteries contain large quantities of esterified cholesterol deriving from the internalization of modified LDL [238]. To mimic the in vivo situation, THP-1 macrophages were preloaded with modified-LDL (ac-LDL). This preloading step stimulated RCT and increased the expression of the cholesterol transporter genes as evidenced by the results from the experiments comparing the negative control treatment (non-preloaded macrophages) with the control (preloaded macrophages) (Fig 2.5 to 2.10). However, the model might be over-stimulated in the sense that the model became sensitive only to the strong agonists and is not able anymore to detect the effect of secondary weak stimuli. Hence, further investigations were carried out to explore this possibility.

Therefore, the effect of c-AMP (the well-known RCT agonist) on ABCA1 gene expression was tested with and without preloading step (Fig 2.12). THP-1 macrophages were incubated with media containing 25 μg/ml ac-LDL (preloaded) or

media without ac-LDL (non-preloaded) for 24 hr prior treatment with c-AMP and ABCA1 gene expression quantified. Firstly, c-AMP with preloading resulted in 1.9-fold increase compared to DMSO with preloading indicating that there was not a problem with c-AMP (Fig 2.12). Interestingly, c-AMP without preloading resulted in 40% reduction in ABCA1 gene expression compared to DMSO with preloading and 130% reduction compared to c-AMP with preloading which shows that c-AMP was not able to induce the RCT process if the macrophages had not been pre-loaded with LDL-cholesterol (Fig 2.12).

Moreover, there was no significant difference between c-AMP without preloading and DMSO without preloading suggesting that the model without the preloading step was not sensitive even to the known agonist. Therefore, preloading with ac-LDL is necessary to ensure the macrophages were converted to foam cells and the RCT process was initiated (Fig 2.12).

Since preloading with ac-LDL is necessary for completion of foam cells formation, it was thought (although it is not likely) that the concentration of ac-LDL used in this experiment was not enough for foam cell process completion and perhaps increasing the concentration of ac-LDL might enhance further the sensitivity of the model. Therefore, THP-1 macrophages were preloaded with 25 and 50 μg/ml of ac-LDL prior treatment to examine the effect of concentration of ac-LDL on stimulation of the model.

In addition, non-preloaded cells treated only with the vehicle DMSO were included to assess the effect of the preloading step as the non-preloaded cells usually give lower gene expression compared to the preloaded cells. The results show that increasing the ac-LDL concentration did not enhance further the sensitivity of the model as there was no statistical differences in ABCA1 gene expression between treatments at either of the two concentrations of ac-LDL tested (Fig 2.13). Moreover, ABCA1 gene expression in non-preloaded cells treated with DMSO was significantly lower than the preloaded cells which mean that the preloading step worked fine and succeeded in stimulating the model. This finding confirmed that the concentration of ac-LDL (25 μg/ml) that had been used in the experiments reported in this chapter was sufficient and there was no need to increase the ac-LDL concentration.

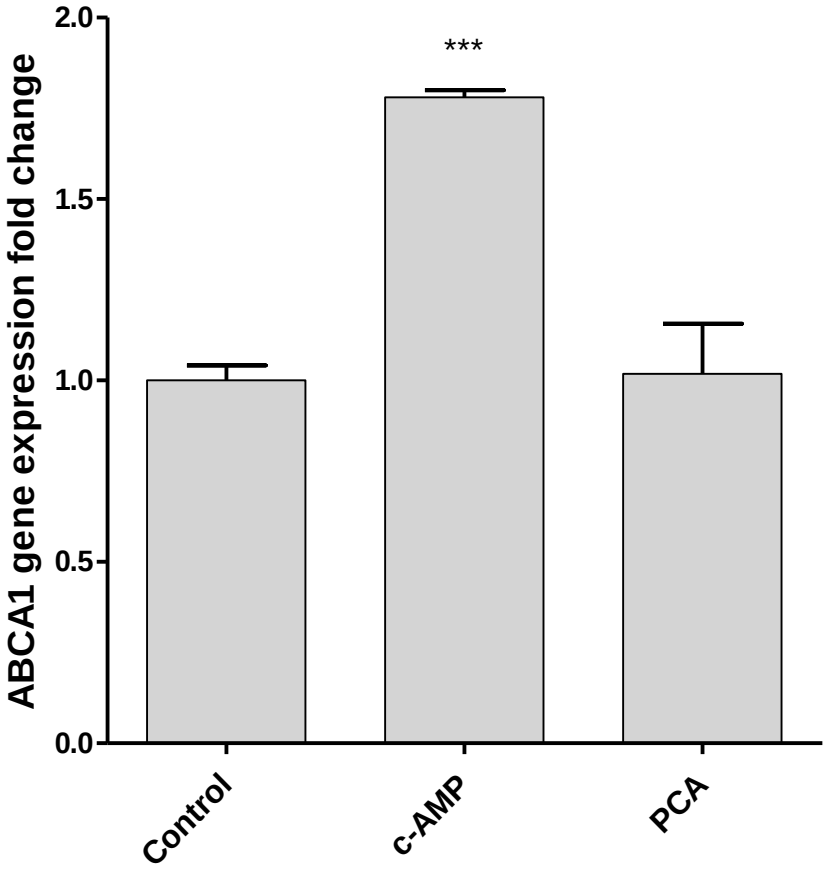


Figure 2. 11: ABCA1 gene expression in THP-1 macrophages stimulated by c-AMP.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, c-AMP= cyclic adenosine monophosphate (300 µM), PCA= 1 µM protocatechuic acid. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

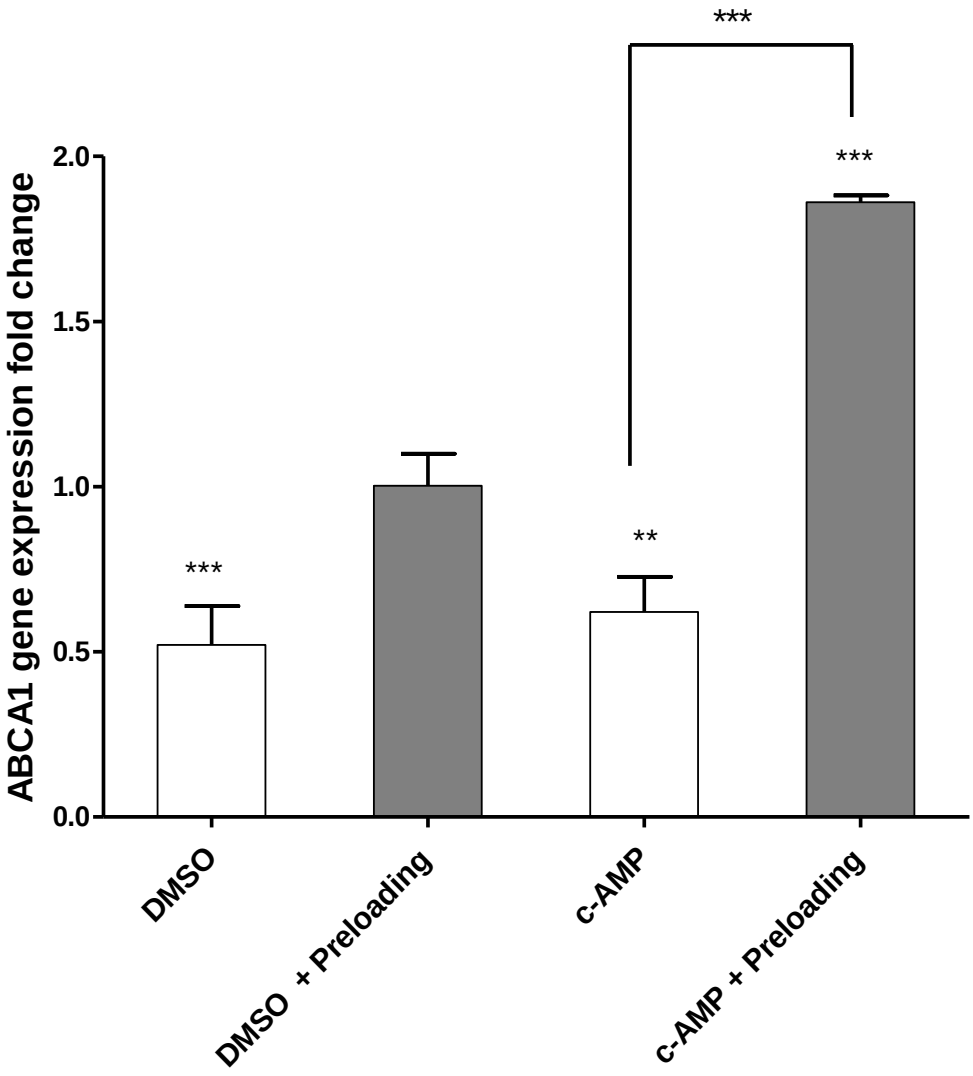


Figure 2. 12: Effect of preloading with ac-LDL on THP-1 model stimulation.

THP-1 derived macrophages were either preloaded with ac-LDL (25 µg/ml) for 24 hr or non-preloaded prior treatment with DMSO (vehicle control) or with c-AMP (300 µM). The fold change was calculated relative to DMSO + Preloading. Data are shown as means ± SD. *** $p \leq 0.001$, ** $p \leq 0.01$ as compared to DMSO + Preloading using one-way ANOVA coupled with Dunnett's multiple comparison test

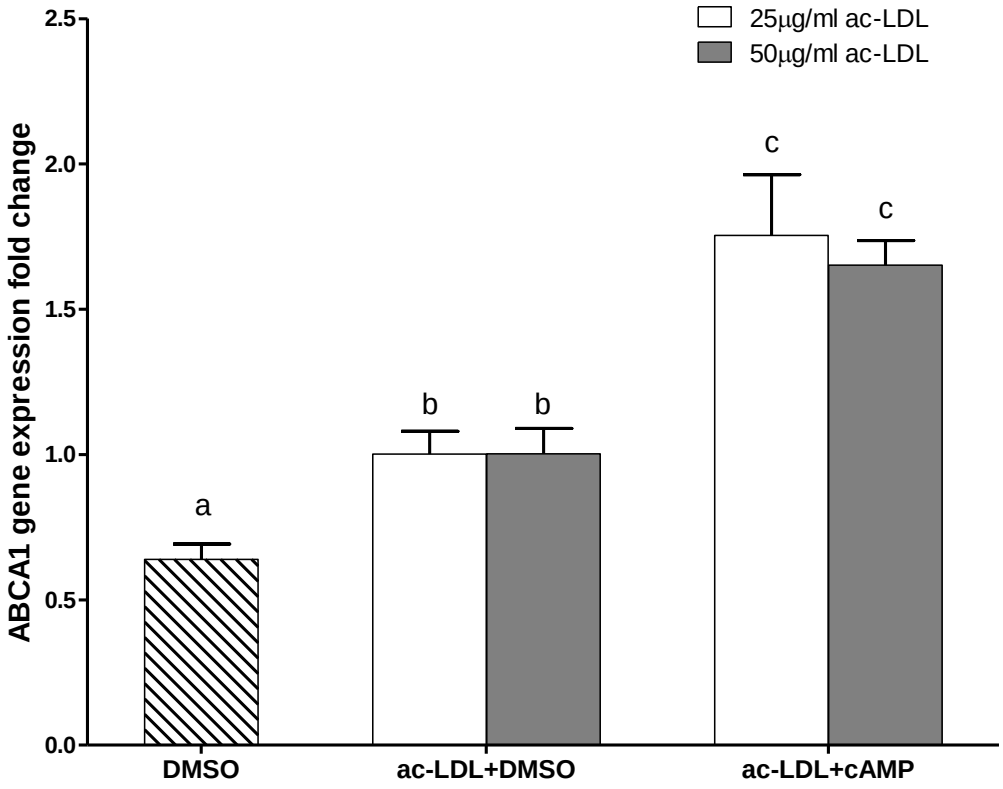


Figure 2. 13: Effect of ac-LDL concentration on THP-1 model stimulation.

THP-1 derived macrophages were preloaded with two ac-LDL concentrations (25 and 50 μg/ml) for 24 hr prior treatment with DMSO (vehicle control) or c-AMP (300 μM). DMSO without preloading treatment has been done by incubating the macrophages with media free from ac-LDL for 24 hr before treatment with DMSO. The fold change was calculated relative to ac-LDL + DMSO. Data are shown as means ± SD. Bars for treatments with different superscript letters are significantly different ($p \leq 0.001$) compared to ac-LDL + DMSO using one-way ANOVA coupled with Dunnett's multiple comparison test.

In the current model, the preloading step (24 h) occurs before exposing the cells to treatments to make sure the cells were converted to foam cells and started to efflux cholesterol. In vivo however, the cholesterol uptake (represented by the preloading step) may occur in parallel to cholesterol efflux (represented by stimulation of cholesterol transporters) to maintain cholesterol homeostasis [219]. Therefore, it was hypothesised that applying treatments simultaneously with the ac-LDL loading may improve the response of the model to putative treatments. To test this, THP-1 macrophages were treated with 25 µg/ml ac-LDL at the same time as the treatment. The treatments were PCA (1 µM), c-AMP and kaempferol and also a control (DMSO). ABCA1 and ABCG1 genes expression were quantified and normalized to control treatment.

As shown in Fig 2.14, PCA did not significantly change the expression of either gene which is consistent with the results presented earlier in this chapter. However, when both treatment and preloading were performed in combination with each other, both c-AMP and kaempferol significantly induced ABCA1 and ABCG1 gene expression (Fig 2.14). But, the magnitude of induction was far less than what was seen when preloading and treatment had been done successively (Fig 2.5 to 2.13). For instance, adding the treatment alongside the preloading reduced the effect of kaempferol on ABCA1 and ABCG1 by 100 and 140%, respectively, compared to the effect when the preloading had been done before the treatment (see Fig 2.14 and Fig 2.5 to 2.10). Similar observations were seen with c-AMP which hardly affected gene expression (Fig 2.14). The reason for this may be that foam cell formation was not complete which meant there was a lower capacity of the cells for cholesterol efflux. This finding confirmed that including a preloading step prior treatment is better for model sensitivity.

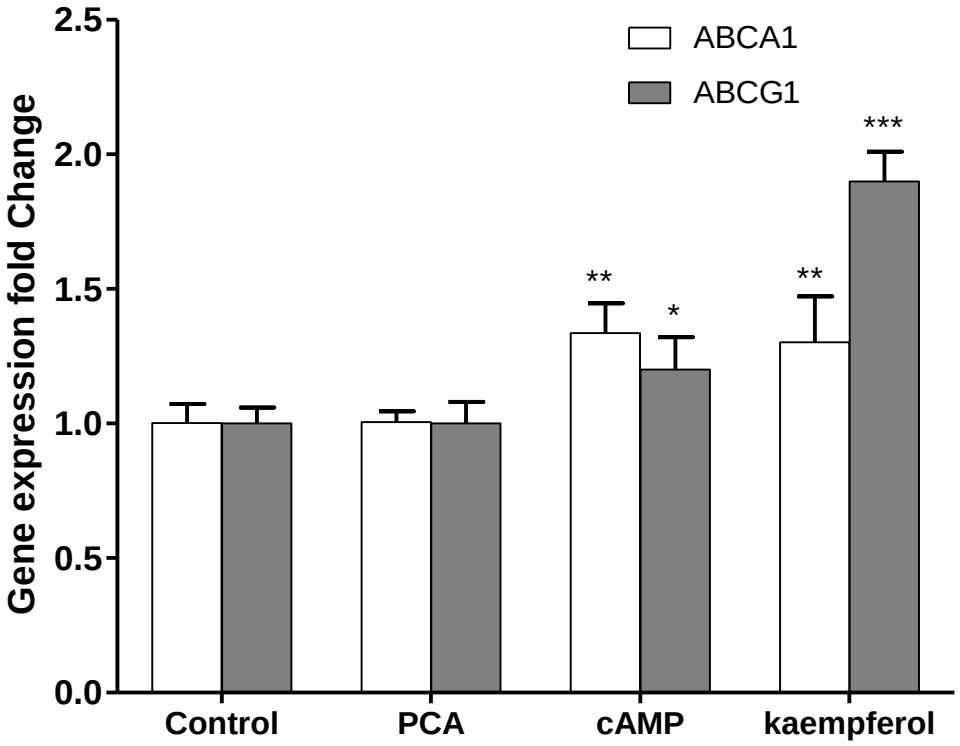


Figure 2. 14: The influence of doing the treatment alongside the preloading step on model sensitivity.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) simultaneously with the treatments for 24 hr. The treatments were control=DMSO, PCA= protocatechuic acid (1 µM), c-AMP = cyclic-AMP (300 µM) and kaempferol (10 µg/ml, 30 µM). The fold change was calculated relative to the control. Data are shown as means ± SD. *** $p \leq 0.001$, ** $p \leq 0.01$ and * $p \leq 0.05$ as compared to the control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.5.2.4.3. The influence of cell passage number

It was recommended that THP-1 should be differentiated at passage numbers lower than 20 to get completely differentiated THP-1 macrophages [238]. In the current model, THP-1 cells were used at passage number from 9 to 15 which is considered a low passage number. However, it is possible that using cells from a lower passage number, even lower than 9, might increase the sensitivity of the model to treatments. Therefore, THP-1 cells were differentiated as previously described at passage number 4. After preloading, differentiated cells were treated with 1 μM PCA and kaempferol. The gene expression of ABCA1 was quantified and normalized to control treatment (DMSO). The analysis showed that there were no significant differences between PCA and the control (DMSO) (Fig 2.15). In addition, kaempferol induced the ABCA1 by 2.4-fold similar to what was previously found when the higher passage numbers had been used (Fig 2.5 to 2.10). Based on that, using passage number lower than previously used has no influence in the model reliability and sensitivity.

2.5.2.4.4. The influence of presence versus absence of fetal bovine serum (FBS)

FBS has been used as a supplement in basal growth medium in cell culture for decades [239]. Therefore, the cell growth, cell differentiation and preloading steps have been performed in media contain 10% FBS. However, lipoproteins found in FBS can bind with polyphenols and suppress their action [240]. Hence, the treatments were performed in FBS-free media which is opposite to what is happening *in vivo* in which macrophages are subjected to the stimuli in the presence of serum. Therefore, conducting the treatments in the presence of FBS was further investigated. After growing, differentiating and preloading THP-1 macrophages in media containing 10% FBS, the cells were then treated with 1 μM PCA, kaempferol and vehicle control (DMSO) using media in the presence or absence of FBS. The -ve (negative control) was non-preloaded cell treated with DMSO. As shown in Fig 2.16 A and B, kaempferol significantly increased ABCA1 and ABCG1 gene expression in both the presence and absence of FBS ($p \leq 0.001$). However, the effect in the absence of FBS was higher than the effect in the presence of FBS (Fig 2.16 A and B). In fact, the effect of kaempferol on ABCG1 in the absence of FBS was two times higher than the effect in the presence of FBS (Fig 2.16 B). Probably, lipoproteins in serum bind with kaempferol and attenuate its action [240]–[242]. Again, no significant changes in ABCA1 and ABCG1 gene expression were observed after PCA treatment using any

of these conditions (Fig 2.16 A and B). These data indicated that conducting the treatment in the presence of FBS negatively influenced the sensitivity of the model, probably because it contains components that can bind the treatment compounds and reduce their efficacy.

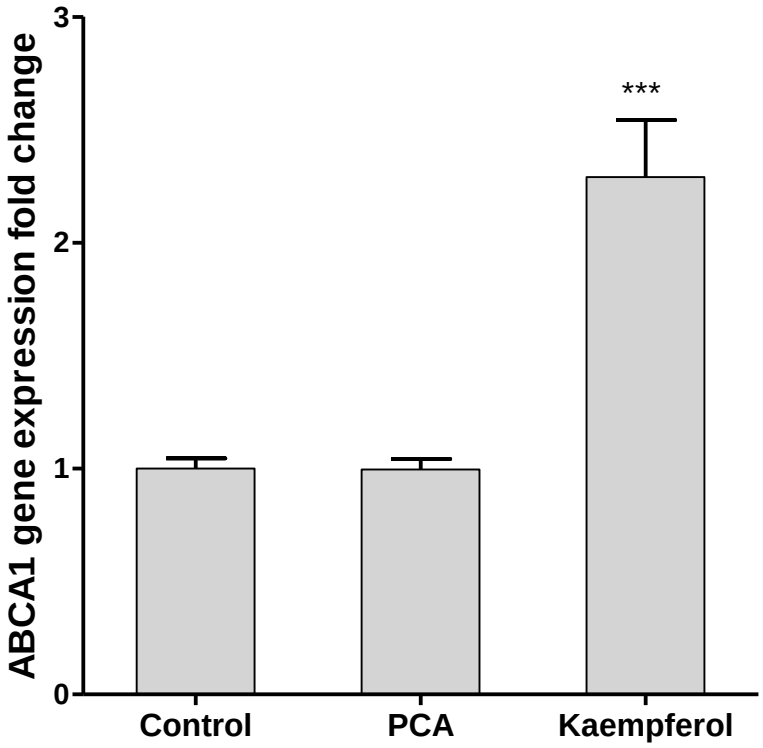


Figure 2. 15: The influence of using THP-1 at low passage number on model sensitivity.

THP-1 derived macrophages at passage number 4 were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, PCA= 1 µM protocatechuic acid and kaempferol (10 µg/ml, 30 µM). The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

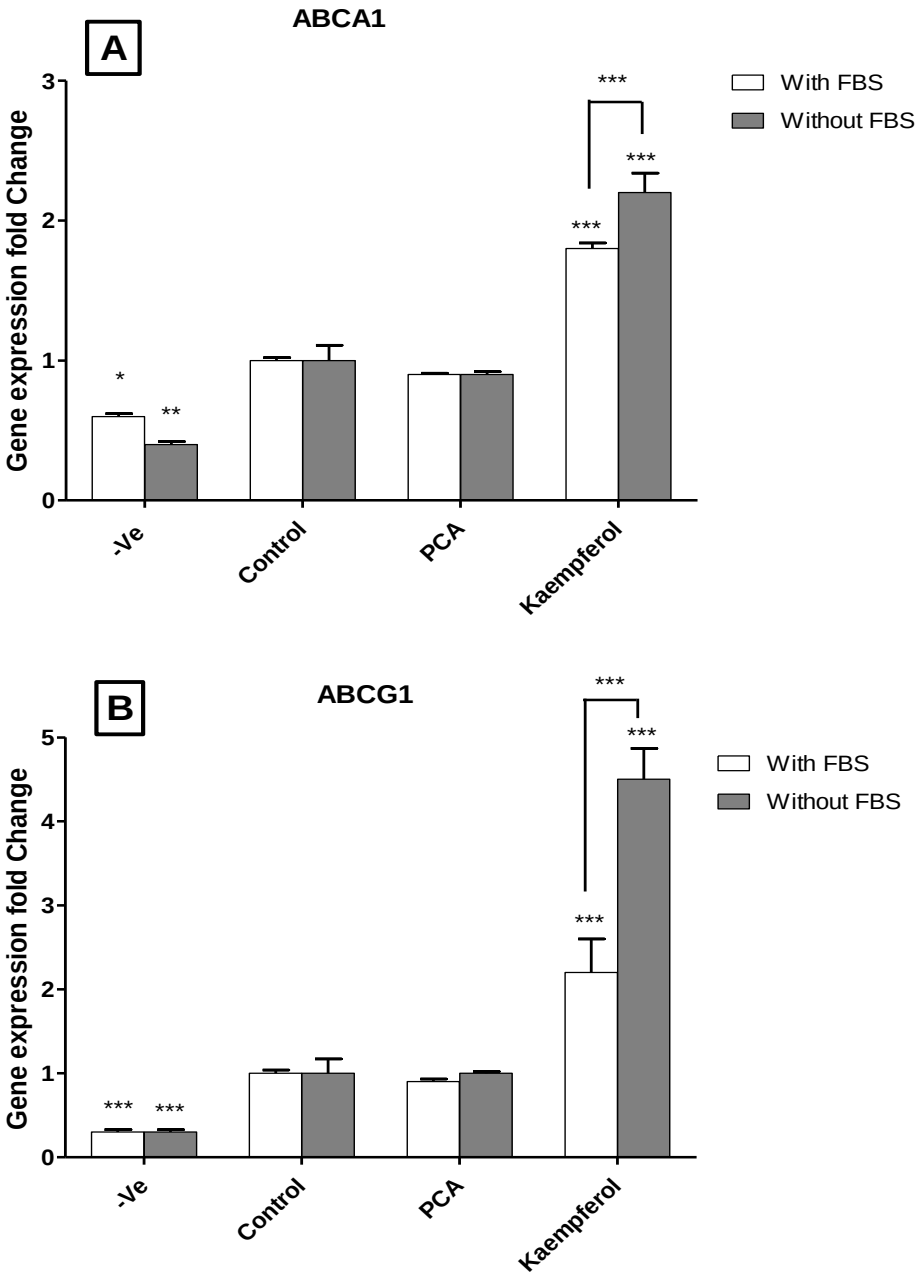


Figure 2. 16: The influence of the presence of fetal bovine serum (FBS) on model sensitivity. (A) ABCA1 gene expression and (B) ABCG1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, PCA= 1 µM protocatechuic acid and kaempferol (10 µg/ml, 30 µM). -Ve is the negative control which is cells treated with DMSO without preloading step. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.5.2.4.5. Using different cell line

Wang *et al.* have previously reported that 1 µM of PCA increased ABCA1 and ABCG1 expression in human (differentiated THP-1) and mouse macrophages (J744.1) [228]. So far work in this chapter has only focused on human macrophages, and so experiments were undertaken to test the effects of PCA on ABCA1 and ABCG1 gene expression in mouse macrophages. J744A.1 mouse macrophages were grown, preloaded as previously described. J744A.1 cells are semi-detach macrophages and does not require a differentiation step. The preloaded J774A.1 macrophages were then treated with 1 µM PCA, c-AMP and the DMSO. No significant changes were observed in ABCA1 gene expression after PCA treatment (Fig 2.17). On the other hand, c-AMP induced ABCA1 gene expression by 1.8-fold ($p \leq 0.001$). This was consistent with data reported earlier in this chapter with human macrophages. These data show that PCA has no effect on cholesterol transporter gene expression in either human- or mouse-derived macrophages.

In summary, from these series of experiments that focused on examining the repeatability, sensitivity and reliability of the model using different conditions and parameters, it has been shown that the current model was working well and produced reliable and repeatable results. It has been shown that the ac-LDL preloading step was necessary to increase the response of the ABCA1 and ABCG1 genes to known effectors, but it was not over stimulated as both the tested positive controls increased both ABCA1 and ABCG1 gene expression. Increasing the ac-LDL concentration did not enhance further the sensitivity meaning that the concentration used was (near) optimal. Applying the treatments simultaneously to the ac-LDL substantially reduced the sensitivity indicating that the order in the current model is the best for model stimulation. No further enhancement was observed with cells at lower passage number, adding FBS to media for treatment reduced the sensitivity and finally, using different cell line did not change the fact that the model produces repeatable results and PCA has no effect on cholesterol transporters gene expression. Based on these findings, anthocyanins and their metabolites did not significantly change the expression of the cholesterol transporter genes ABCA1 and ABCG1 in THP-1-derived macrophages.

Having eliminated ABCA1 and ABCG1 as putative targets for anthocyanins and / or their metabolites, the search for other putative targets in the RCT pathway were investigated. The first was cellular cholesterol uptake.

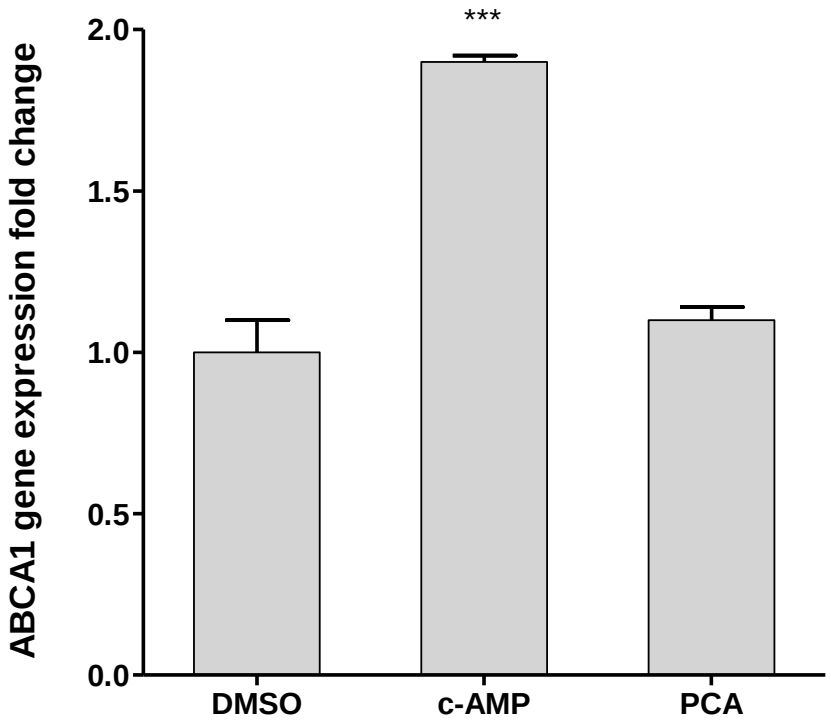


Figure 2. 17: ABCA1 gene expression in J774A.1 treated with PCA and c-AMP.

J774A.1 macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, c-AMP= cyclic adenosine monophosphate (300 µM), PCA= 1 µM protocatechuic acid. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.5.2. The *in vitro* effect of anthocyanins and their metabolites on the expression of genes involved in cholesterol uptake

The scavenger receptors (SR) play a vital role in meditating the uptake and internalization of modified-LDL that initiates foam cell formation [225]**.** Inhibiting the expression of SR may attenuate foam cell formation [224]. Therefore, the effect of anthocyanins and their metabolites on the gene expression of three members of the SR family (MSR1, SCARB1 and CD36) were investigated. The preloaded THP-1-derived macrophages were treated with 10 µM of C3G and D3G for 24 hr as representative anthocyanin parent compounds. To investigate the effect of anthocyanin metabolites, the cells were treated with 10 µM of PCA, PGA, ferulic acid, vanillic acid, 4HBA and gallic acid for 24 hr. The gene expression of MSR1, SCARB1 and CD36 were quantified using RT-qPCR.

For anthocyanin parent compounds, C3G and D3G slightly increased the expression of the MSR1 and SCARB1 genes (Fig 2.18-2.19), although the effect was not significant. The expression of CD36 was not changed compared to control (Fig 2.20). Unexpectedly, PCA at a concentration of 10 µM significantly increased the expression of the MSR1 gene by 1.4-fold change (Fig 2.18, $p \leq 0.05$). Furthermore, PCA caused a small but non-significant increase in expression of the SCARB1 gene (Fig 2.19), whereas PCA did not change the expression of the CD36 gene (Fig 2.20). No significant changes were observed in the expression of any of the three genes after 4HBA treatment (Fig 2.18-2.20). No statistically significant changes were observed with other treatments.

Based on these results, there was not a direct interaction between anthocyanins and their metabolites with genes driving either cholesterol transporter or cholesterol uptake in the in vitro model used in the previous experiment. However, a secondary effect of anthocyanins on these genes is not excluded. Therefore, exploring the effects using an in vivo model was suggested. Therefore, the gene expression of cholesterol transporters and cholesterol uptake were investigated in tissues isolated from mice treated with anthocyanins.

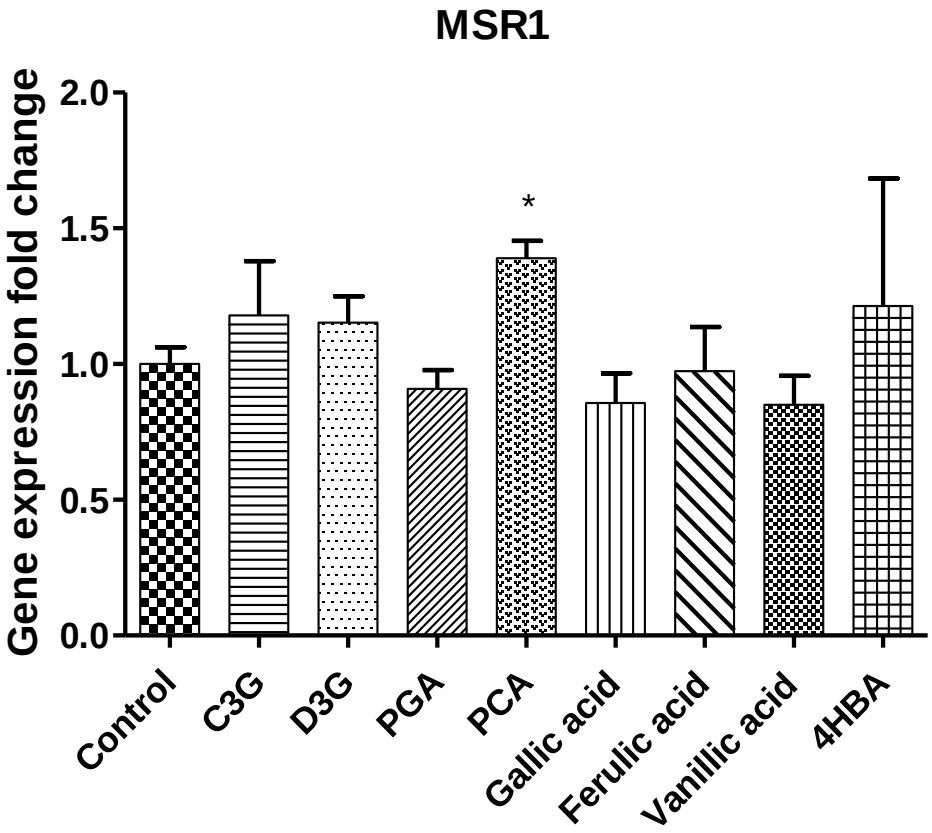


Figure 2. 18: Effect of anthocyanins and their metabolites on MSR1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, C3G = cyanidin-3-glucoside, D3G = delphinidin-3-glucoside, PGA = phloroglucinaldehyde, PCA = protocatechuic acid and 4HBA = 4-hydroxybenzaldehyde. The fold change was calculated relative to control. Data are shown as means ± SD. * $p \leq 0.05$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

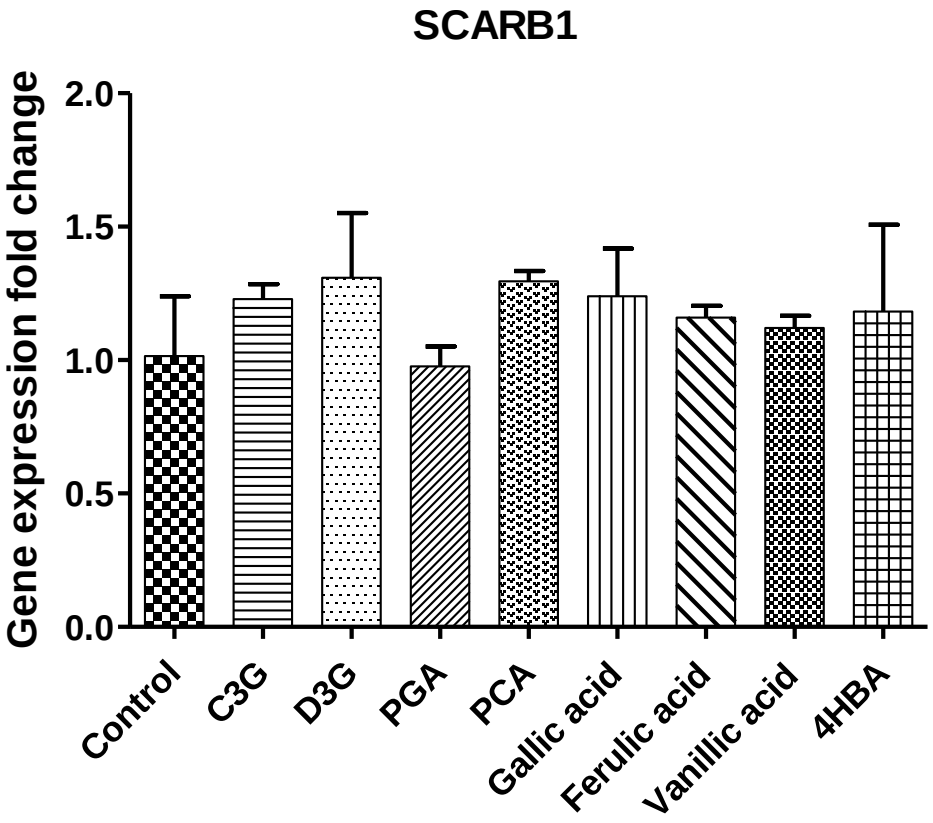


Figure 2. 19: Effect of anthocyanins and their metabolites on SCARB1 gene expression.

THP-1 derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, C3G = cyanidin-3-glucoside, D3G = delphinidin-3-glucoside, PGA = phloroglucinaldehyde, PCA = protocatechuic acid and 4HBA = 4-hydroxybenzaldehyde. The fold change was calculated relative to control. Data are shown as means ± SD. No significant differences detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test.

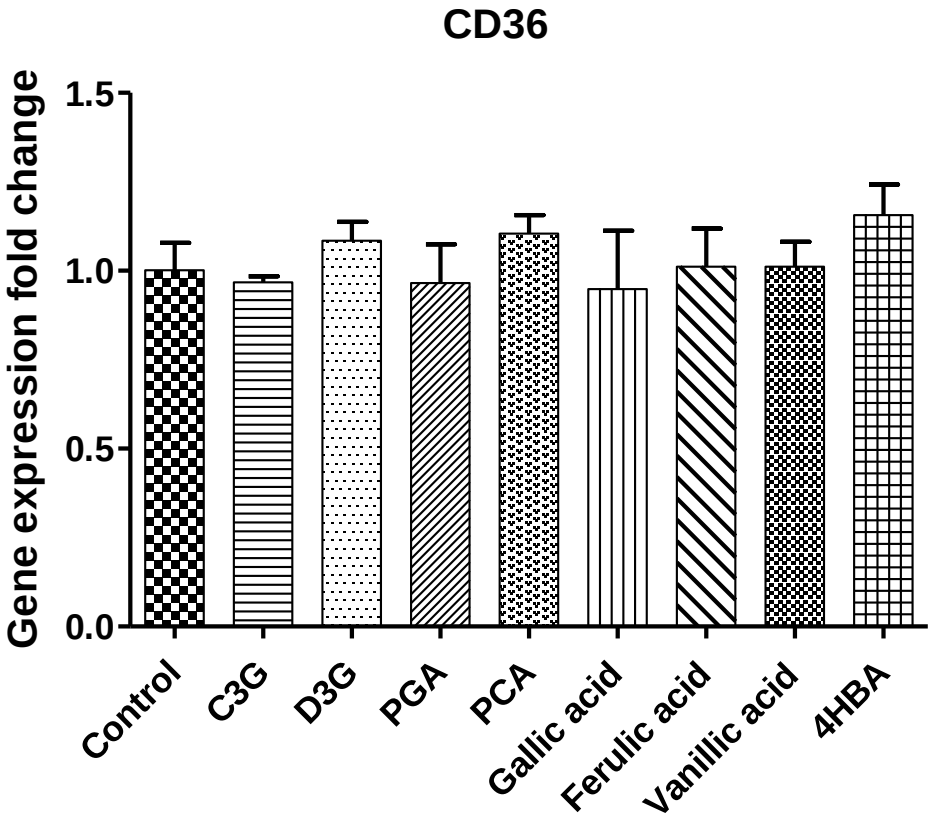


Figure 2. 20: Effect of anthocyanins and their metabolites on CD36 gene expression.

THP-1-derived macrophages were preloaded with ac-LDL (25 µg/ml) for 24 hr prior treatment. Control = DMSO, C3G = cyanidin-3-glucoside, D3G = delphinidin-3-glucoside, PGA = phloroglucinaldehyde, PCA = protocatechuic acid and 4HBA = 4-hydroxybenzaldehyde. The fold change was calculated relative to control. Data are shown as means ± SD. No significant differences detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.5.3. The effect of anthocyanins consumption on the gene expression of cholesterol transporters *in vivo*.

To test the *in vivo* effect of anthocyanins on ABCA1 and ABCG1 gene expression (the cholesterol transporters), tissues from ApoE$^{-/-}$ mice that were fed high-polyphenol tomatoes were used. Four groups of mice were fed four types of tomatoes that were high in either flavonols, a mixture of anthocyanins and flavonols (ANCs + Flavonols), resveratrol or isoflavones. The effects of consumption of these different types of high-polyphenol tomatoes were compared to control group in which the mice were fed normal red tomatoes (low polyphenol tomato). The animal intervention was conducted by Mark Winterbone. I only completed the analysis of the relative gene expression in the liver and aorta tissues. I undertook the removal of the fat from the tissue and further tissue processing, the extraction of RNA from aorta and liver tissue samples, and the analysis of gene expression.

With regard the outcome of the animal feeding study, the primary outcome was the size of plaques in the aortic arch. This data indicated that the diets supplemented with tomatoes expressing a mixture of anthocyanins and flavonols and the tomatoes expressing resveratrol caused reductions in atherosclerotic plaque size in mouse aortic sinuses by 31 and 26%, respectively (data unpublished), compared to the diet supplemented with control red tomato. Since removal of cholesterol from plaques is a key process underpinning reduction in plaque size, it was hypothesised that RCT key genes in these two groups would be altered by the diets.

For ABCA1 and ABCG1 gene expression in aorta, ANCs + Flavonols tomatoes increased ABCG1 gene expression by 1.4-fold compared to the control tomatoes, although this was not significant, while, ABCA1 remained unchanged (Fig 2.21 A and B). Resveratrol tomatoes increased both ABCA1 and ABCG1 by 1.4- and 1.3-fold, respectively; however, the increases were not significant. Surprisingly, ABCA1 gene expression was significantly increased by 1.9-fold following consumption of isoflavone tomatoes, whereas flavonol tomatoes non-significantly increased ABCG1 by 1.7-fold (Fig 2.21 A-B, $p \leq 0.05$). According to these findings, anthocyanin-containing tomatoes did not influence ABCA1 and ABCG1 gene expression in aortas which is consistent with the results obtained from the *in vitro* work with cultured macrophages. For liver tissues, ANCs + Flavanols tomatoes did not alter ABCA1 and ABCG1 gene expression compared to the red control tomatoes (Fig 2.22 A and B). Unexpectedly, isoflavone-expressing tomatoes significantly reduced ABCA1 by 25% (Fig 2.22 A, $p \leq 0.05$). No significant changes were observed with other treatments.

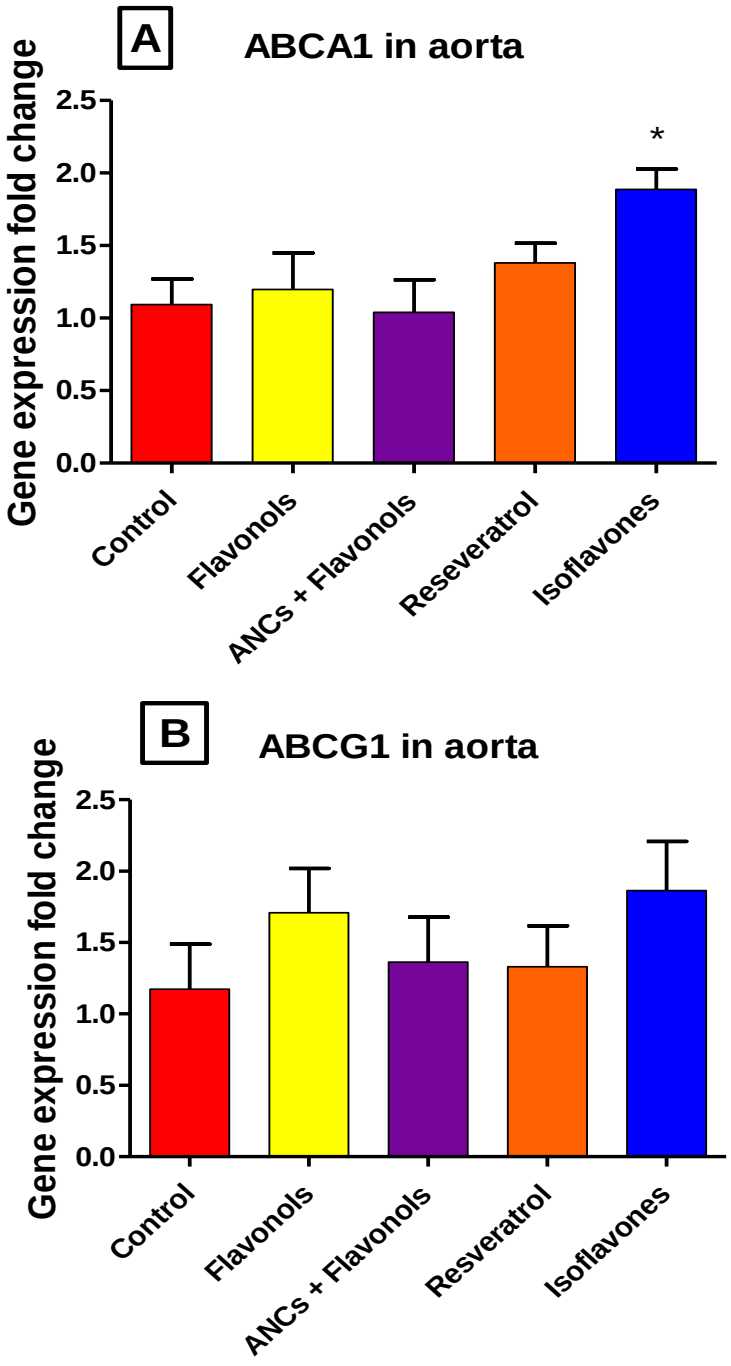


Figure 2. 21: The gene expression of (A) ABCA1 and (B) ABCG1 in aorta tissue isolated from *Apo E*$^{-/-}$ mice fed with high-polyphenol tomatoes.

Control = red tomatoes (polyphenol-free tomatoes), ANCs + Flavonols = a combination of anthocyanins and flavonols. The fold change was calculated relative to control. Data are shown as means ± SD. * $p \leq 0.05$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

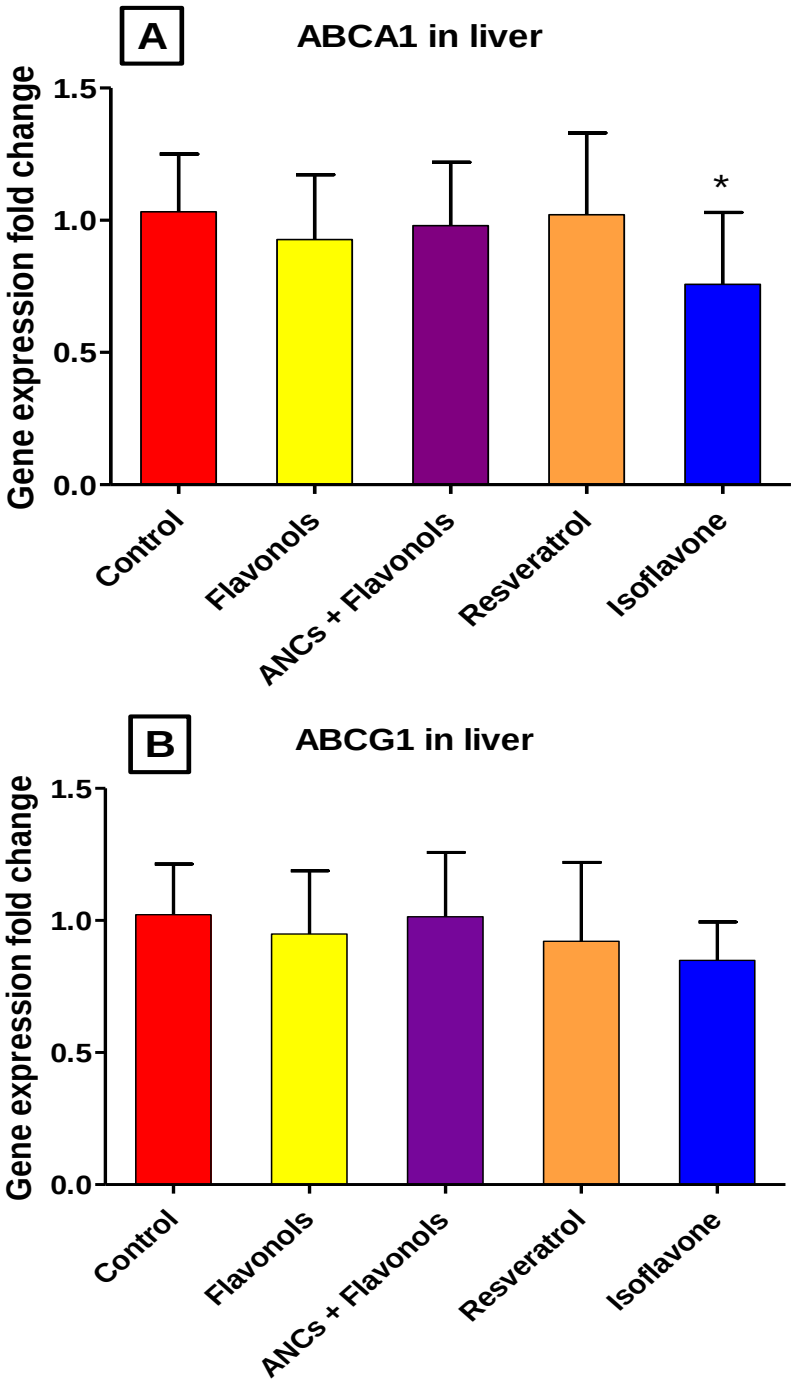


Figure 2. 22: The gene expression of (A) ABCA1 and (B) ABCG1 in liver tissue isolated from *Apo E-/-* mice fed with high-polyphenol tomatoes.

Control = red tomatoes (polyphenol-free tomatoes), ANCs + Flavonols = a combination of anthocyanins and flavonols. The fold change was calculated relative to control. Data are shown as means ± SD. * $p \leq 0.05$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.5.4. The effect of anthocyanins consumption on the gene expression of cholesterol uptake *in vivo*

The gene expression of scavenger receptors MSR1, SCARB1 and CD36 in aorta, were quantified using the same aorta RNA extracts that had been used in analyses described in the section above. None of the polyphenol-rich tomato groups significantly influenced MSR1 or SCARB1 gene expression, although the consumption of isoflavone-expressing tomatoes slightly increased MSR1 gene expression (Fig 2.23A, B). Anthocyanin-containing tomatoes slightly increased CD36, albeit this was not significant. Similarly, flavonols and resveratrol tomatoes increased CD36 gene expression, but this was not significant (Fig 2.23C)

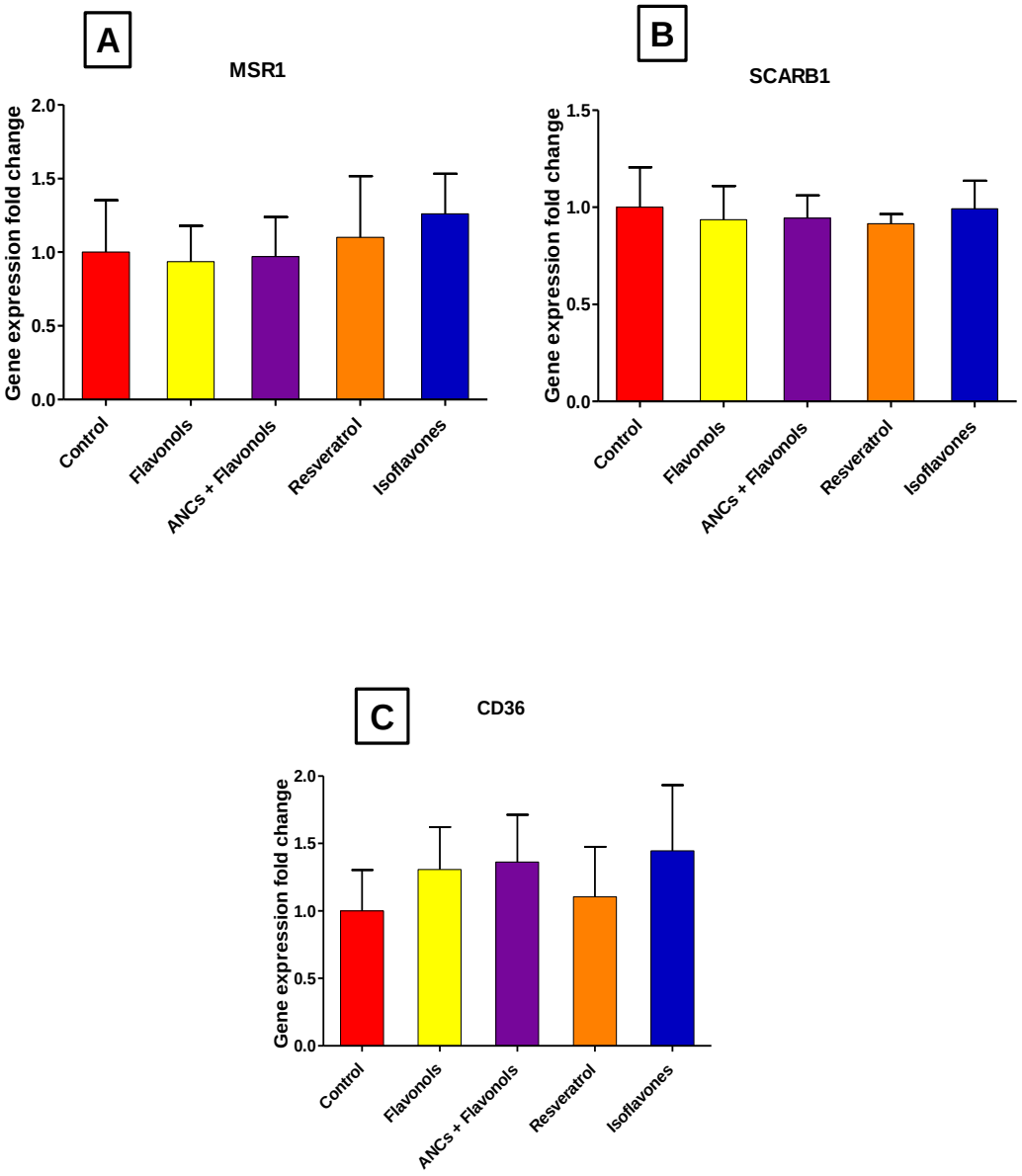


Figure 2. 23: The gene expression of (A) MSR1, (B) SCARB1 and (C) CD36 in aorta tissue isolated from *Apo E-/-* mice fed with high-polyphenol tomatoes.

Control = red tomatoes (polyphenol-free tomatoes), ANCs + Flavonols = a combination of anthocyanins and flavonols. The fold change was calculated relative to control. Data are shown as means ± SD. * $p \leq 0.05$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test.

2.6. Discussion

Data from dietary interventions with human participants and in animal models has shown that anthocyanins have the potential to modify circulating lipoprotein profiles, specifically by reducing total and LDL-cholesterol levels and increasing the levels of HDL-cholesterol [164]. However, the mechanisms behind this effect remain elusive: First, it is not known whether the anthocyanin parent compounds per se or one or more of the myriad of smaller phenolic acid-type metabolites that interact with cells and tissues to alter lipoprotein metabolism, and second it is not known where in the pathway they act (i.e. with which components of the RCT pathway and in what manner). The in vitro work with cultured macrophages described in this chapter represents a fairly comprehensive investigation to find out which anthocyanins/metabolites were responsible and with which components of the RCT pathway they interacted. This research goes well beyond the current state-of-the-art which is limited to a single report describing increases in the expression of the ABCA1 and ABCG1 cholesterol efflux transporters in human and mouse-derived macrophages by PCA [228].

The main findings were that (1) none of the two major anthocyanin parent compounds or their metabolites significantly affected the expression of cholesterol transporters genes (ABCA1 and ABCG1); (2) none of the parent compound of anthocyanins or their metabolites altered the gene expression of the scavenger receptors (MSR1, SCARB1 and CD36), although PCA slightly increased the gene expression of MSR1.

The data presented in this chapter contradict with what Wang *et al.* 2012 has previously reported [228]. They reported that PCA but not C3G increased ABCA1 and ABCG1 by 2.6 and 4 fold, respectively which support the idea that anthocyanin metabolites are more beneficial [228]. However, the data in this chapter did not support this notion. In the current study, C3G did not affect the gene expression which was consisted with the previous report. However, PCA did not alter the gene expression of either ABCA1 and ABCG1, although the model used in this experiment was very similar to the model used by Wang *et al.* (2012) [228]. The only difference could be that they used an isolated ac-LDL made inhouse, while a commercially available ac-LDL was used in the current study. Probably (although it is unlikely), the isolated ac-LDL that they used contains other molecules that affect the response to the treatment. Another possibility could be using serum free media in treatment preparation. It is not mentioned whether FBS-free/containing media was used in Wang *et al*. (2012) paper and may be this condition affect the respond. I tried to

contact the authors to find out what is the conditions they used but I have not got any response. It was obvious that the current model used in this chapter was highly sensitive and the data was reliable as both the positive controls and negative control worked well.

Apart from C3G and PCA [228], the effect of all other tested metabolites in this study on RCT gene expression were investigated for the first time. The pharmacokinetics study of isotope-labelled C3G [73] showed that PCA wasn't the main metabolite of C3G but ferulic acid, vanillic acid and hippuric acid were the main ones. The reported concentration of PCA in human plasma was 146 nm, while the concentration of vanillic acid, ferulic acid, hydroxybenzaldehyde were 1845, 827 and 667 nm, respectively which is higher than PCA by at least 4.5 to 12-fold [73]. However, the effect of these metabolites on ABCA1 and ABCG1 remained unexplored. In the previous report [228], it was totally ignored that PCA is subject to phase II conjugation and the effects of PCA conjugates has not been investigated yet. In addition, the fact that anthocyanin metabolites are found in mixture and there is high possibility of synergistic effect was unexplored. Moreover, no one has investigated the effect of D3G and its potential metabolites and there is a gap of knowledge which needs further investigation. Therefore, the effect of two major anthocyanin types, C3G and D3G, and 18 of their metabolites including their recently identified human metabolites of C3G and the predicted metabolites of D3G on the gene expression of key elements of RCT have been investigated for the first time. Most of these metabolites have been recently identified and their biological effects are still relatively unknown which make the data presented here novel. To the best of my knowledge, this is the first *in vitro* study to examine the effects of the pure synthetic phase II conjugates of anthocyanins on RCT at physiologically relevant concentrations. This is the first *in vitro* study to include the concept that anthocyanin metabolites are present in mixtures and they may synergistically operate to exhibit their effects. Moreover, one of the main strengths of the work presented in this chapter was the concentrations of the tested metabolites. This study examined the effects of anthocyanins and their metabolites at two different physiologically-relevant concentrations, 1 µM and 10 µM which were within the peak concentrations of anthocyanin metabolites found in human plasma after consumption of ^{13}C-labelled C3G (ranged from 0.1 - 2 µM, with cumulative concentrations of metabolites reaching 10 µM) [73], [75].

The reason for using THP-1 cell as a model to investigate the underlying mechanisms by which anthocyanins and their metabolites exhibit their effect was due to the vital role that human macrophages play in atherosclerosis. Foam cells found in

atherosclerotic vascular lesion are derived from macrophages [243]. Like in the case of blood monocyte-macrophages, THP-1 cells increase in number in monocyte stage and it can be easily differentiated chemically into macrophage stage in which the cells stop dividing [244]. Thereby, THP-1 cells were used in this model. THP-1 monocytes were originated from the peripheral blood of a leukemic one-year-old male. THP-1 can be differentiated into macrophages by using PMA. After differentiation, THP-1 convert to adherent cells and stop dividing [245]. To mimic physiological status of the cholesterol-laden macrophages (foam cells) which contain large quantities of esterified cholesterol, THP-1-derived macrophages should be preloaded with a modified LDL such as ac-LDL. Ac-LDL increased the quantity of esterified cholesterol by several folds in THP-1 macrophages [238]. Therefore, ac-LDL has been used in the preloading step to stimulate the model. The data in this chapter clearly showed the preloading step was successful and the foam cell formation process was completed as there was a clear significant difference in the gene expression of preloaded and non-preloaded cells (negative control).

Despite the fact that the model used in this study is sensitive to treatments, the contradiction with Wang et al. 2012 lead to further examination of the sensitivity of the current model at different conditions and the use of different parameters to confirm the repeatability and reliability of the results obtained. Firstly, both kaempferol and c-AMP, the well-known ABCA and ABCG1 agonists, increased the gene expression of ABCA1 and ABCG1 similar to what have been previously reported [236], [237] indicating that the model was sensitive. Additionally, changing the conditions of the preloading reduced the sensitivity of the model even to the well-known agonists suggesting that the current conditions of preloading step were optimum. Furthermore, the passage number that used in this model has no influence on the model sensitivity. It can be concluded from this further optimisation that the current model is sensitive to treatments and the model successfully worked with two different cell lines.

Since none of the anthocyanins or their metabolites affected the gene expression of cholesterol transporters, the effect on the gene expression of scavenger receptors, another element of RCT, were investigated. Scavenger receptors are responsible for internalization of modified-LDL[246]–[248]. Scavenger receptors have different families according to their structure and therefore they can bind with different ligands in addition to LDL derivatives. However, in this study, only three members that belong to family A and family B which mainly bind with LDL and its derivatives as a ligand were investigated [225]. These three members were: (1) MSR1 (also known as SR-AI), (2) SCARB1 (also known as SR-BI) and CD36. These three receptors were

chosen as they all can use ac-LDL which was used in preloading step as a ligand. PCA increased MSR1 gene expression by 1.4-fold change. Otherwise, none of the tested metabolites changed the gene expression of any of selected scavenger receptors. Although it was expected to observe a reduction in gene expression, there was non-significant slight increase on MSR1 with some metabolites on MSR1. Probably because the chemical differentiation and preloading steps that used in THP-1 model. Several studies have reported an increase in the expression of scavenger receptors in THP-1 cell after treatment with PMA and ac-LDL which used in the differentiation and preloading step [238].

There are several explanations that may explain why the tested metabolites did not show any effects. One reason (although it is unlikely) could be that the more effective metabolites have not been tested [73], [91]. Although, wide range of anthocyanin metabolites were tested in this study, there are other compounds have not been tested. Phenylacetic acid and 3-(4-hydroxyphenyl) propionic acid are good example for newly discovered metabolites that should be considered in future work [94]. Another example, in our lab, high concentration of homoprotocatechuic acid (3,4 dihydroxyphenyl acetic acid) was identified for the first time after incubation of C3G with human faeces from a donor using the colon model (unpublished data). The differences in microbiota was suggested as a reason for producing this new compound. For instance, in ^{13}C-labelled C3G study which conducted with 8 participants [73], vanillic acid was identified only in two participants, while, the other six participants did not produce it which means that the metabolisms differ from one person to another or from one status to another and there are un-identified metabolites which may contribute to the biological activity of anthocyanins. Therefore, it is expected that different metabolites may be derived by the action of different microbiota and one of these compounds might be responsible for the biological activity of anthocyanin. Therefore, exploring the newly discovered metabolites would explain the biological activity of anthocyanins. Another explanation is that the interaction between anthocyanin metabolites play the crucial role and they interact all together to deliver the action of anthocyanins. Due to time and cost, only a mixture of some but not all C3G and D3G metabolites were examined. The mixture of C3G metabolites were analysed separately from the mixture of D3G metabolites, while, they are found together in food and their metabolites are present together in plasma. Is possible that if all C3G and/or D3G metabolites were mixed together, an effect on cholesterol transporter genes might be observed.

Although, there was not any significant effect observed in RCT key genes in vitro, anthocyanins might target different pathways and different cell lines. For instance, Krga et al. (2018) found that the anthocyanins and their gut metabolites downregulated the monocyte adhesion and trans-endothelial migration pathways in HUVECs cell line [249]. Similarly, anthocyanin metabolites significantly reduced IL-6 protein levels and VCAM-1 protein production in HUVECs [250]. IL-6 and VCAM-1 play a key role in the progression of atherosclerosis [250]. Therefore, more work is still required to investigate more pathways.

Since it is possible that the reported changes in circulating lipoprotein profiles in dietary intervention studies in human participants [164] and animal models [251], [252] are caused by secondary effects rather than direct interactions between anthocyanins/metabolites and components of the RCT pathway such as scavenger receptors and cholesterol efflux transporter proteins, the effects of dietary interventions with anthocyanins was also investigated. It is challenging to determine the specific mechanisms if effects are caused by a secondary rather than primary effect. Possible routes for secondary effects include via the gut microbiota (e.g. the anthocyanins/metabolites alter the structure and function of the gut microbiome which in turn affects lipoprotein metabolism, perhaps by changing bile acid metabolism and rates of enterohepatic recirculation). It should be noted that investigating possible secondary effects was not within the scope of this thesis chapter or indeed the PhD project. Therefore, the effect of consuming anthocyanin-expressing tomatoes on the gene expression of cholesterol transporters and scavenger receptors were investigated in aorta and liver tissues isolated from $ApoE^{-/-}$ mice.

In this intervention, the mice fed with genetically modified tomato enriched in different type but the same concentration of polyphenols. It was found in this intervention that flavonols + anthocyanins containing tomatoes and resveratrol tomatoes significantly reduced the size of the atherosclerotic plaque in mouse aortic sinuses by 31 and 26%, respectively (unpublished data). After quantifying the gene expression of key elements of RCT in the liver and aorta, the main findings were that (1) anthocyanin-expressing tomatoes did not significantly changed the gene expression of ABCA1 and ABCG1 in aorta and liver, although a slight non-significant increase was observed in ABCG1 gene expression in aorta and (2) anthocyanin-containing tomatoes did not significantly affect the scavenger receptors gene expression in aorta. It should be noted that anthocyanin-containing tomatoes express both anthocyanins and flavanols. Isoflavones-containing tomatoes increased ABCA1 in aorta. However, the impact of this effect is not understood as the same treatment did not change the lipid

profile in the plasma or in the liver tissue and reduced ABCA1 gene expression in liver. Further investigation is still required to understand the effect of isoflavones on lipid metabolism.

In summary, PCA did not affect the expression of ABCA1 or ABCG1 gene which contradicts a single report in the literature. The data presented in chapter described a comprehensive study of the effects of all the main human metabolites of C3G and the known and predicted metabolites of D3G and have demonstrated that none of these have any significant effect on the expression of the two macrophage cholesterol efflux transporters or on the scavenger receptors that mediate cholesterol uptake by macrophages. In addition to the in vitro data, the possible effects of anthocyanin-supplemented diets versus anthocyanin-free control diets on the expression of the cholesterol efflux transporter genes in aortic and liver tissues was quantified and saw no significant differences, even though the anthocyanin-supplemented diets had caused favourable changes in circulating lipoprotein profiles in the animals.

As mentioned elsewhere in this chapter that RCT has different key steps and therefore, anthocyanins and their metabolites may target different level such as HDL function. Therefore, the effect of anthocyanins and their metabolites on paraoxonase 1 (PON1) (marker of HDL function) was investigated and presented in chapter three.

2.7. Conclusions.

Taken together, the data presented here do not support the notions that the effects of anthocyanins on lipoprotein metabolism are caused by interactions between anthocyanin parent compounds or one or more of their phenolic-type metabolites with key transporters involved in the RCT process both in vitro and in vivo. However, it doesn't exclude the possibility that other untested or unidentified metabolites would affect the gene expression. Future work should consider testing more anthocyanins metabolites individually and in mixtures that contain all anthocyanins metabolites. Both the *in vitro* and *in vivo* data did not exclude the possibility that anthocyanins would target another level of RCT such as HDL function and therefore the next chapter will explore the effect of anthocyanins and their metabolites on PON1 (marker of HDL function) gene expression and activities.

CHAPTER THREE

Chapter three: The effects of anthocyanins and their metabolites on PON1 as a marker of HDL function

3.1. Abstract

High HDL quantity and functions are inversely associated with CVD risk. Paraoxonase 1 (PON1) contributes to many atheroprotective functions of HDL such as promoting the reverse transport of cholesterol process and reducing the levels of oxidized LDL. PON1 activities are influenced by several factors, most importantly, diet and genetic polymorphisms. Randomized controlled trial data indicates that anthocyanin consumption increased PON1 activity. However, the underlying molecular mechanisms by which anthocyanin induce PON1 activity are not understood. In the current study, it was proposed that anthocyanins may increase PON1 through upregulation of gene expression or as activator for the enzyme itself. Therefore, the aim of this research was to investigate the ability of anthocyanins and their metabolites to increase PON1 gene expression and enzyme activities. The effect of the two predominant dietary anthocyanins and 18 of their recently identified microbial metabolites including their phase II conjugates on PON1 gene expression was studied using a PON1-Huh7 stably-transfected cell line and reporter gene assay. The effects of these compounds on PON1 arylesterase and lactonase activities was investigated using two isoforms of the PON1 enzyme that are the phenotyps of the 192Q/R polymorphism (PON-RR and PON-QQ). None of the compounds caused significant changes in PON1 promoter activity ($p \geq 0.05$). further, none of the compounds caused significant changes in arylesterase and lactonase activity regardless of the phenotype at physiological concentrations. However, cyanidin reduced the lactonase activity of PON-RR at high concentrations but not at physiologically-achievable concentrations. In conclusion, no evidence was found that anthocyanins and/or their metabolites could affect PON1 gene expression or enzyme activity. Future work should focus on investigating the effects of anthocyanins on LDL oxidation status and PON1 secretion, and to consider other atherosclerotic-related pathways.

3.2. Introduction

More recent studies have suggested that the relationship between HDL and CVD markers extends beyond the concentration of HDL alone and that the function of HDL may be more important than HDL concentration in protecting against CVD diseases [253]. HDL possesses a number of atheroprotective functions such as mediating cholesterol efflux, protecting against oxidation and inflammation, and promoting nitric oxide [220].

HDL can protect LDL from oxidation and therefore attenuate atherosclerosis development by preventing the production of oxidized LDL. This protective effect could be attributed to enzymes associated with HDL including paraoxonase 1 (PON1) [254]. PON-1 has been the focus of research activities because of its capacity to stimulate cholesterol efflux and protect HDL and LDL from oxidative modifications, thus inhibiting atherosclerosis [255].

3.2.1. Paraoxonase 1 (PON1)

PON1 is an HDL-associated enzyme secreted by the liver and found to have significant anti-oxidative and anti-inflammatory properties through its lactonase, peroxidase and esterase activities [256]. Low PON1 activity has been found in numerous pathological conditions associated with atherosclerosis, such as diabetes, hypercholesterolaemia, obesity and metabolic syndrome [257]. It also found that PON1 knockout mice showed increased levels of plaque formation and oxidative stress in atherosclerotic lesions as well as having altered anti-inflammatory activity of HDL. This advocates PON1 as a potential anti-atherosclerotic agent [258]. The antiatherogenicity of PON1 was suggested to be related to its ability to hydrolyse oxidized cholesteryl esters, oxidized phospholipids, phosphatidylcholine core aldehydes, and degrade hydrogen peroxide, thus protecting lipoprotein particles from any further oxidative modification [259].

3.2.2. PON1 activities

PON1 has different enzyme activities based on the substrate used. PON1 can be evaluated as paraoxonase when the substrate used is paraoxon from which PON1 got its name [260]. It can be referred to as an arylesterase (when arylesters such as phenyl acetate or p-nitrophenyl acetate are used as substrates) or as diazoxonase (when diazoxon is used as substrate) or as lactonase (when lactones are used as substrate) [261]. Despite the fact that PON1 was traditionally described as an arylesterase/paraoxonase and its activity was measured accordingly, lactonase has

been recently shown to be PON1's native activity [254]. Impairing the lactonase activity of PON1, e.g. via mutations of its catalytic dyad, diminished the ability of the enzyme to prevent LDL oxidation, reduced HDL-mediated cholesterol efflux and abolished lysophosphatidylcholine (LPC) formation from phospholipid in macrophages suggesting that the actual biological antiatherogenic functions of PON1 may be mediated by its lactonase activity [262]. PON1 lactonase activity was also reported to hydrolyse homocysteine thiolactone, a toxic metabolite of homocysteine, which has been reported to be involved in homocysteine-induced vascular damage [263].

Because arylesterase and paraoxonase are possibly not physiologically relevant activities, whereas lactonase is the native physiologically relevant activity, it is important to study PON1 as a lactonase when investigating its role in antiatherogenesis. However, the majority of studies haven't focused on lactonase activity and therefore more studies are still required using appropriate substrates such as lipophilic lactones.

3.2.3. Paraoxonase polymorphism

Nearly 200 single nucleotide polymorphisms (SNPs) have been identified in the human PON1 gene. However, the SNPs that alter amino acids located at position 55 (leucine/L or methionine/M) and at the position 192 (glutamine/Q or arginine/R) of PON1 are more associated with lipoprotein oxidation and CHD risk and seem to be more involved in the differences in PON1 activity between individuals [264]. People with the 192-Q/Q genotype gain greater protection against CVD. The 192-Q/Q genotype is associated with high HDL and decreased level of cholesterol, LDL, triglyceride and apo B protein [265]. The 192- Q/Q enzyme was more potent than R/R in decreasing the levels of oxidized lipids in human atherosclerotic lesions [266]. The PON1 L55M polymorphism has also been associated with variation in serum PON1 activity levels but has a weaker effect [267]. In a healthy non-diabetic population, both PON1 activity and specific activity are affected by the L55M polymorphism [265]. The effect of polymorphisms also varies depending upon the substrate leading to different phenotypes. For instance, the 192- R/R polymorphism hydrolyses paraoxon approximately 9 times faster than 192- Q/Q, while the opposite happens when diazoxon and sarin were used as a substrate [268]. Thereby, conducting a comparison between individuals regardless of the differences in genotypes/phenotypes could lead to a false interpretation, especially, when substrates that are strongly influenced by polymorphisms such as paraoxon are being

used [260]. Therefore, it is recommended to compare between values of PON1 levels within each different genotype/phenotype instead of general comparison of all population.

There are many other PON1 polymorphisms that have been reported to be associated with risk of CVD and other chronic diseases. For instance, rs 3735590 at the 3′ UTR of the PON1 gene which is within a miRNA binding site was reported to be associated with ischaemic stroke and thinner carotid artery intima-media thickness (IMT). This SNP affects the binding of the negative regulator miRNA-616 which inhibits PON1 expression [269]. Another example is the SNP that is located at the -107 position of the promoter region of the PON1 gene. It is referred as T (-107) C and was reported to affect both PON1 gene expression and serum activity [270].

Although the wide range of PON1 activity among individuals is usually interpreted as genotype differences, there are additional environmental factors that could modulate PON1 activity, and diet and lifestyle are the major ones among those factors [271]. For instance, vitamins A, E, and C supplementation restored the decreased paraoxonase activity [272]. Furthermore, there is an interaction between PON1 genotype and the environmental factors such as diet. It was reported that there is a significant association between anthocyanin intake and increased HDL levels in 4 from 18 independent tagging SNPs in the PON1 gene which have been linked to lipid profiles and cardiovascular risk [273].

3.2.4. Effect of polyphenols on paraoxonase 1

Several dietary intervention studies in humans and animals have reported that polyphenol consumption has promising effects on PON1 activity. This was shown in a study by Zhu *et al.* (2014) in which a total of 122 hypercholesteraemic human subjects were given 320 mg/day anthocyanins or placebo (n = 61 per group) for 24 weeks. They reported that anthocyanin consumption increased the activity of HDL-PON1 by 17.4% compared with placebo [170]. Aviram *et al.* (2000) observed a similar effect on serum PON1 in humans after they fed pomegranate juice (1.5 mmol total polyphenols per day) for two weeks [274]. On the other hand, quercetin consumption did not significantly change PON1 activity in serum collected from 35 healthy normal-weight volunteers after two weeks supplementation with doses of 50, 100 and 150 mg/day [275].

In animal studies, polyphenol consumption has been linked to improved PON1 activity. For instance, Fuhrman *et al.* (2002) found that supplementation with 50

µg/day catechin and quercetin for 6 weeks increased serum PON1 activity by 113% and 75%, respectively in Apo $E^{-/-}$ mice [276]. This increase might be attributed to the upregulation in PON1 gene expression in liver as the hepatic PON1 mRNA increased by 2-fold after 2 weeks consumption of quercetin using C57BL/6 mice [275]. In another dietary intervention study in 28 male rats, the consumption of soy isoflavones for 8 weeks significantly increased plasma PON1 activity [277].

In addition to studies in humans and animals, several cell model experiments also have shown positive effects of polyphenols on PON1 activity and gene expression. For instance, Esatbeyoglu, *et al.* (2017) studied the effect of polyphenols derived from purple sweet potatoes (PSP) which were fractionated into two fractions, anthocyanin- and copigment-rich fractions. Treatment of PON1-Huh7 cells with PSP-copigment-rich fraction resulted in significant dose-dependent induction of PON1 transactivation, while PSP-anthocyanins-rich fraction was a weak inducer [278]. In the previous study [278], a mixture of phenolic compounds was tested and it is difficult to identify which phenolic compound was responsible for the observed increase. However, many studies have also been conducted using pure phenolic compounds to investigate their effect on PON1. Gouedard *et al.* (2004) studied the effect of resveratrol, which belongs to a class of polyphenolic compounds called stilbenes found predominantly in grape skin. The report describes the effects on PON1 enzymatic activity, gene expression and PON1 promoter activity using Huh7 cell line. They reported that 10 µM resveratrol for 48 hr resulted in a significant increase of both PON1 secretion (62%), cell-based enzyme activity (50%) and gene expression (> 2-fold). They also found that PON1 promotor activity was increased in a dose-dependent manner [279]. These findings have been confirmed in HepG2 cells by Gupta *et al.* (2014) who reported that resveratrol increased PON1 secreted enzymatic activity in a dose-dependent manner with highest activity at 15 µM, and also increased the gene and protein expression of PON1 [280]. In another study, resveratrol induced PON1promotor activity by 3.4-fold compared with control [275]. The effect of flavonols such as quercetin and its methylated derivative isorhamnetin on PON1 have also been investigated. Isorhamnetin induced a 2.7-fold increase in PON1 promotor activity, while, quercetin increased PON1 promotor activity by 1.4-fold as compared to control [275]. Quercetin (10 µM) also increased the cell-based PON1 activity by 50% in Huh7 cell line [281]. Gouedard *et al.* (2004) tested the effect of other phenolics on PON1 gene expression in Huh7. They found that naringenin, flavone, catechin and quercetin increased PON-1 mRNA by about two fold but that catechin was a poor

inducer [281]. Conversely, Mahrooz *et al.* (2011) reported that 10 µM naringenin inhibited both paraoxonase and arylesterase of purified human PON1 activity [254].

In summary, data in the literature shows that some phenolic compounds could act as PON-1 inducers. However, only two human intervention studies which were conducted with Medox™ [170] pomegranate juice [274], and one *in vitro* study that used PSP-anthocyanins [278] have reported the effect of anthocyanins on PON1 [278]. The Zhu *et al.* (2014) study was the only report that reported the effects of purified anthocyanins on PON1 serum activity in humans [170]. Nevertheless, they used a proprietary product, Medox™, which is a combination of 17 different natural anthocyanins which did not allow any conclusion to be drawn about which anthocyanin or anthocyanins were responsible. Moreover, they completely ignored the influence of polymorphisms in PON1 which would mean a range of unknown phenotypes within the study population. Additionally, none of these studies have investigated the effects of anthocyanin metabolites or investigated how anthocyanins mediated the increase in PON1 activity. Therefore, further investigation is still needed to explore the effects of anthocyanins and their metabolites on PON1 gene expression and activities with considering the differences between PON1 phenotypes.

3.3. Objectives

The overall aim of this chapter was to explore the molecular mechanisms by which anthocyanins induce PON1 activity as a marker of HDL function. The specific objectives of the research described in were to: (1) investigate the effect of anthocyanins and their metabolites on PON1 gene expression and (2) investigate the effect of anthocyanins and their metabolites on arylesterase and lactonase activities using two different PON1 phenotypes.

3.4. Materials and methods

3.4.1. Reporter gene assay

The effect of anthocyanins and their metabolites on PON1 gene expression was evaluated using a reporter gene assay (Bioluminescence firefly luciferase assay) in cultured hepatocytes. PON1-Huh7 is a Huh7 liver hepatoma cell line that had been stably transfected with a reporter plasmid containing 1009bp [-1013, -4] of the PON1 gene promoter cloned into the firefly luciferase reporter vector pGL3 basic. The luciferase reporter vector is commonly used to study gene expression and any other post-translational modifications [275], [282]. Briefly, once the PON-1 promoter is activated (e.g. by treatment), the reporter gene (luciferase gene) will be expressed and therefore, luciferase enzyme will be produced (Fig 3.1). The activity of the luciferase enzyme can be then easily measured by using a chemiluminescence assay of a protein extract. The more the treatment has the capacity to induce PON1 promoter, the more luciferase activity can be obtained (Fig 3.1). The light intensity reflects the gene expression which is a function of the promoter activity.

3.4.1.1. Cell culture

PON1-Huh7 cells were kindly provided by Prof Gerald Rimbach, Institute of Human Nutrition and Food Science, University of Kiel, Germany. The cells were maintained following the protocol as previously described [282]. Briefly, PON-Huh7 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Cat. # D5671) supplemented with 10% FBS (v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Thermofisher, Cat# 15140122), glutamine 2 mM (Sigma-Aldrich, Cat. # 59202C) and 100 µg/ml G418 disulphate (Sigma-Aldrich, Cat. # G8168) at 37°C in 5% CO_2 until reaching 80-90 % confluency with the media changed every two days. The cells were then detached by adding trypsin-EDTA (Thermofisher, Cat# 25300054) for 2-3 min at 37°C. A complete DMEM that contain 10% FBS was then added to inhibit the trypsin. The trypsinised cell suspension was collected and the cells were either split or seeded for treatments. In 24-well plates, cells were seeded at an initial density of 0.15 x10^6 cells/well and left to attach for 24 hr. After that, the media was removed and new pre-warmed media containing anthocyanins treatment was added to the appropriate wells and incubated at 37 °C, 5% CO_2 for 48 hr.

3.4.1.2. Treatments

In this experiment, the same metabolites that were tested in the previous chapter were used. The treatments were prepared as previously described earlier in section 2.4.2.

The only differences are that (1) the treatments were prepared in complete DMEM growth media containing 10% FBS and (2) 20 µM of curcumin (Fisher Scientific, Cat# AC218580100) was used as a positive control alongside the anthocyanins and their metabolites treatments. After treatment, the luciferase activity was measured as a marker of PON1 promoter activity. Treatments were conducted in quadruplicate and the experiments were repeated at least two times.

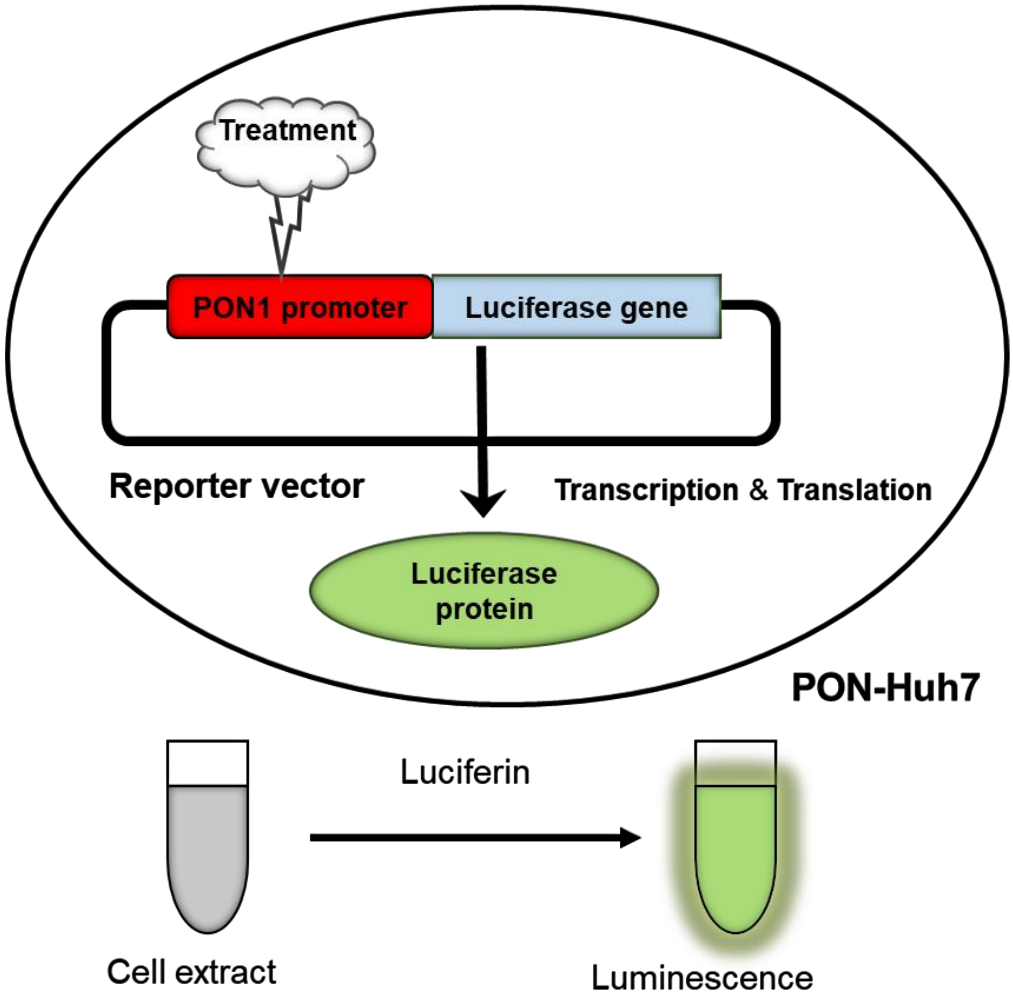


Figure 3. 1: Principle of a simple reporter gene assay.

The reporter plasmid vector contains the target promoter sequence and a luciferase gene. After triggering the promoter, the luciferase gene will be expressed in the living cells and produce luciferase protein which catalyzes a reaction with luciferin to produce light. The amount of expressed luciferase protein can be estimated from measuring the light intensity which reflects the promoter activity in living cells.

3.4.1.3. Luciferase assay

Luciferase activity was measured using the Luciferase Assay System (Promega, Cat# E1500) according to the manufacturer's instructions. Briefly, after 48 h treatment, the media was removed and the cell layer was washed twice with cold calcium- and magnesium-free PBS (Thermofisher, Cat# 14190094). The lysis buffer was then added to the cell layer and the cells were scraped. Immediately, the lysate was collected and centrifuged for 2 min at 12,000 XG at 4ºC. After that, in 96-well white plates, 100 µl of luciferase assay reagent was add to 20 µl cell lysate. The luminescence was immediately measured over 20 sec using a plate reader; FLUROstar Optima (BMG labtech, UK). The plate reader is capable of injecting the reagent automatically and can perform multiwell reading. The luminescence was expressed as total light intensity which was collected over 20 sec. Results were normalised to the total cell protein content. The total protein in each sample was determined using the bicinchoninic acid (BCA)-Reducing Agent Compatible assay as per manufacturer's protocol (Thermofisher, Cat# 23252).

3.4.1.4. PON1 promoter activity calculation

First, the light intensity which reflect the promoter activity was normalized to the total protein content in the cell lysate.

Promoter activity = Total light intensity / total protein content (µg/ml)

The fold change of PON1 was calculated relative to the control (DMSO).

3.4.2. PON1 enzyme activities

The direct effect of anthocyanins on PON1 activities has been measured using a commercially available purified PON1 that has been phenotyped into two phenotypes based on Q192R polymorphism. PON1 phenotype QQ (PON-QQ) and phenotype RR (PON-RR) were purchased from ZeptoMetrix (Cat# 0801384). In this experiment, the same metabolites that tested in the previous chapter were used. The treatments were prepared as previously described earlier in section 2.4.2. The only difference is that the treatments were diluted in the assay buffer. Arylesterase and lactonase PON1 activities were measured using colourimetric assays as described below.

3.4.2.1. PON1 arylesterase

PON1 arylesterase activity was quantified by measuring the hydrolysis rate of p-nitrophenyl acetate, the colourless substrate, into p-nitrophenol that has yellow colour and measuring the increase in absorbance using a spectrophotometer (Fig 3.2 A) [283]. PON1 arylesterase was measured as described elsewhere [254], [284], [285].

First, PON-RR and PON-QQ were diluted in assay buffer consisting of 20 mM Tris-HCl buffer, pH=8 and 1 mM $CaCl_2$ just prior to conducting the assay. The dilution factors for PON-RR and PON-QQ were 10- and 15-fold, respectively. These two dilutions were chosen after a series of dilutions were tested to obtain a linear phase of the enzyme reaction.

Just prior the assay, the treatments were prepared and diluted in the assay buffer. While diluting the enzymes and treatments, it was considered that a further dilution will occur in the assay plate when the treatments will mix together with enzyme, substrate and the assay buffer. Briefly, the assay was developed with a final volume of 200 µl in 96-well polystyrene plate. 20 µl of diluted enzyme and 20 µl of treatment mix together with 140 µl pre-warmed assay buffer. The plate was then sealed to prevent evaporation and incubated at 37°C for 10 min. Just prior to adding the substrate, the stock solution of p-nitrophenyl acetate (0.5M) (Sigma-Aldrich. Cat# N8130) was diluted into 5 mM in pre-warmed assay buffer. Quickly, 20 µl of diluted substrate was added to the previous reaction mixture and the increase in absorbance was measured Immediately at 410 nm for 10 min using FLUROstar Optima (BMG labtech, UK). So, the final concentration of reactants would be 1% PON-RR / 0.7% PON-QQ, 1 mM p-nitrophenyl acetate and 1 or 10 µM anthocyanins treatments. The final concentration of DMSO was 0.1% in all treatments. The control treatment was enzyme treated with DMSO at final concentration of 0.1%. The slope of the reaction rate was calculated using the instrument software. Blanks without enzymes or treatments were used to correct for the spontaneous nonenzymatic hydrolysis of p-nitrophenyl acetate by subtracting blanks from the hydrolysis rate of treatments. The % of change was calculated relative to the control as following:

$$\%\ of\ Chang = \frac{Rate\ of\ treatment - Rate\ of\ control}{Rate\ of\ control}\ x\ 100$$

Treatments were conducted in triplicates and the experiments were repeated at least two times. The purity of the enzymes was determined by measuring the activity of enzymes with 100 µM of 2-hydroxyquinoline (2-HQ), the PON1-potent inhibitor (Sigma-Aldrich, Cat# **270873).**

3.4.2.2. PON1 lactonase

PON1 lactonase activity was measured spectrophotometrically by monitoring the hydrolysis of the synthetic lipolactone substrate, 5-thiobutyl butyrolactone (TBBL) [286], [287]. TBBL was synthesised inhouse using the method described elsewhere [288]. The principle of this assay is that the enzyme releases a colourless thiol moiety

upon hydrolysis of the oxo-lactone ring of TBBL, the colourless substrate (Fig 3.2 B). The release of thiol can be detected by adding a chromogenic probe such as 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) that produces a yellow colour with thiol. Therefore, the enzyme rate can be measured by monitoring the increase in absorbance after adding DTNB to the reaction mixture (Fig 3.2 B).

Lactonase activity was measured as described previously [288], [289]. Briefly, PON-RR and PON-QQ were diluted to 50- and 80-fold, respectively in the assay buffer that consisted of 50 mM Tris-HCl, 1 mM $CaCl_2$, 50 mM NaCl and pH=8. These two dilutions were chosen after a series of dilutions were tested to obtain a linear phase of the enzyme reaction. The treatments were prepared as previously mentioned in arylesterase section (3.8.2.1). Using 96-well polystyrene plates, 20 µl of enzyme and 20 µl of treatment were mixed together with 60 µl pre-warmed assay buffer. 50 µl of DTNB (4 mM) was mixed with the reaction mixture. The plate was sealed to prevent evaporation and incubated for 10 min at 37°C. A solution of TBBL (2 mM) was prepared in pre-warmed assay buffer contain 2% acetonitrile just before use and 50 µl of TBBL (2 mM) was added to the reaction mixture and the absorbance was measured immediately at 412nm using a FLUROstar Optima plate reader (BMG labtech, UK). The final volume was 200 µl and the concentration of reactant were 0.2% PON-RR / 0.125% PON-QQ, 1 mM DTNB, 0.5 mM TBBL and 1 or 10 µM treatments. The lactonase activity was calculated as described in the arylesterase section (3.8.2.1). Treatments were conducted in quadruplicate and the experiments were repeated at least two times. The purity of the enzymes was determined by measuring the activity of enzymes with 100 µM of 2-hydroxyquinoline (2-HQ).

3.4.3. PON1 gene expression and enzyme activity in HepG2 cell.

3.4.3.1. Cell culture

Human hepatoma cell line HepG2 was maintained in minimum essential medium with Earle′s Balanced Salts (EMEM) containing 2mM L-glutamine (Sigma-Aldrich, Cat# 51411C) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) in 75T flask at 37°C in a humidified incubator with atmosphere containing 5% CO_2.

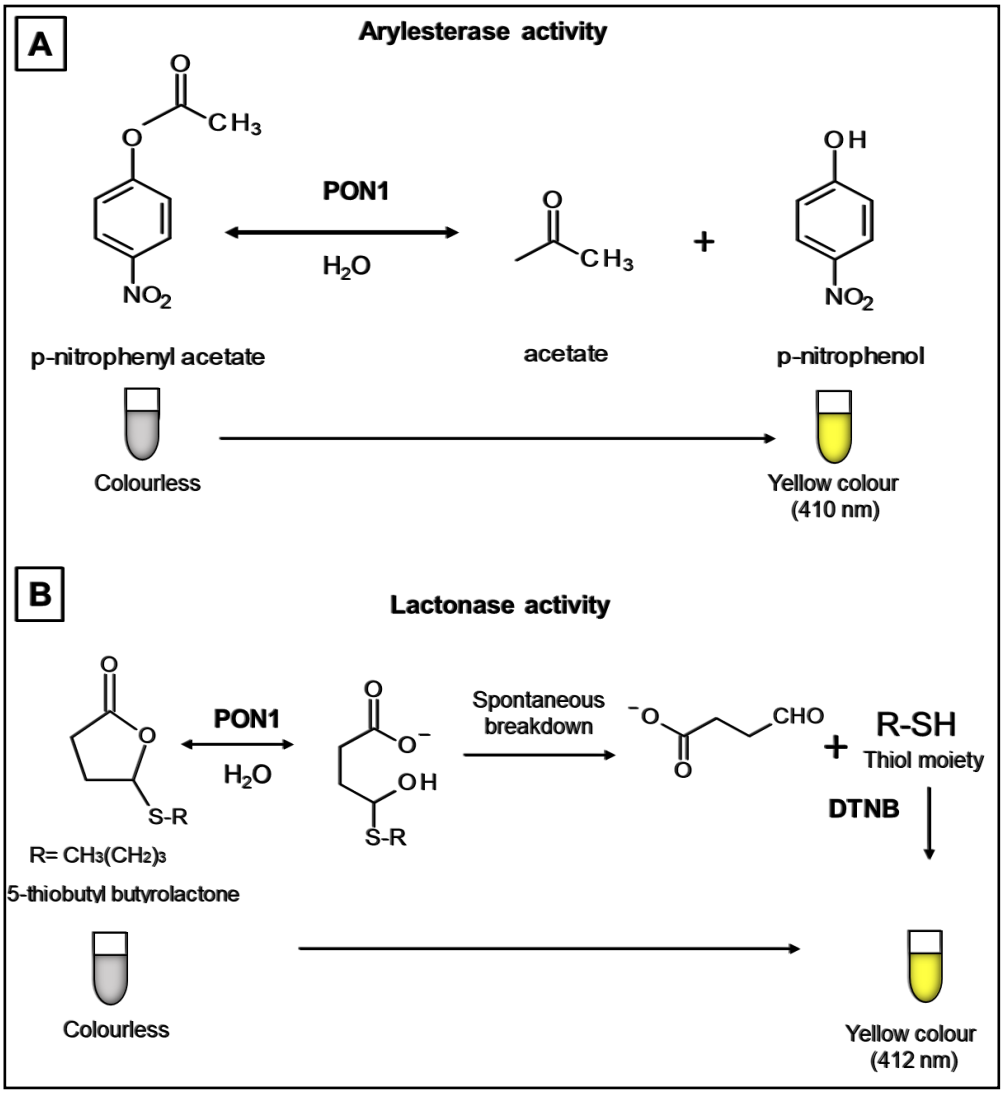


Figure 3. 2: The principle of measuring PON1 (A) arylesterase and (B) lactonase activities.

The figure demonstrates the hydrolysis of p-nitrophenyl acetate (arylesterase substrate) and 5-thioalkyl butyrolactones (lactonase substrate) by PON1 enzyme. DTNB =5,5′-Dithiobis (2-nitrobenzoic acid).

3.4.3.2. Secreted PON1 activity

HepG2 were seeded in 6-well plates (0.5 x 10^6 cell/well) using complete media for 48 hr. After that, the medium was removed, and cells were washed twice with pre-warmed PBS. The cell layer was then treated with compound of interest using serum-free medium for 48 h. The media, then was collected and used as source of PON1 enzyme. PON1 arylesterase activity was measured as described early in section 3.8.2.1.

3.4.3.3. Cell-based PON1 activity

The cells were seeded as previously described. After 48 h, the cells were incubated with compound of interested prepared in media for additional 48 h. At the end of the incubation period, the media was discarded, and the cells then were washed twice with pre-warmed PBS. Cell-based PON1 activity was measured by adding the reaction mixture that contain the substrate to the cell layer and monitoring the change in absorbance. The reaction was initiated by the addition of 1 ml of buffer containing p-nitrophenyl acetate to yield final concentration of 1mM. The reaction mixture was incubated at 37°C and the absorbance was recorded at 410 nm at the end of 20 min incubation. At the same condition,1 ml of reaction mixture containing 100 µM 2-HQ was added to number of treated wells to correct for PON-1 specific activity. Autohydrolysis background activity was also determined by the addition of reaction mixture to cell-free well

3.4.3.4. PON1 gene expression in HepG2

The cells were treated with DMSO and 10 µM of PCA as early mentioned. RNA extraction and qRT-PCR reaction were performed as described in section 2.4.3 and 2.4.6. PON1 gene expression was normalized against GAPDH. The sequences of forward and reverse primers were: 5`-TTCA CCCGATGGCAAGTATG-3` and 5`-ACGAGGGTATTAAAGTCAAGGG-3`, respectively.

3.4.4. Statistical analysis

All data and statistics were analysed using GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). All values are given as means ± SD. Any statistical difference between the groups was determined with one-way ANOVA coupled with Dunnett's multiple comparison test comparing all sample groups to control (DMSO 0.1%). Values of $p \leq 0.05$ were considered significant.

3.5. Results

3.5.1. PON1 activity and gene expression in HepG2 cell

Because the liver is the major source for circulating PON1, the best model to study the effect of anthocyanins on PON1 activity is to use human liver hepatocellular cells such as HepG2. Toward the goal of developing an assay to test the effect of anthocyanins and their metabolites on PON1 expression, activity and secretion, the ability of HepG2 to express and secrete PON1 was tested.

3.5.1.1. Secreted PON1 activity.

To investigate whether HepG2 secrete PON1 in to the media, HepG2 cells were treated with 10 μM PCA and DMSO (both prepared in FBS-free media) for 48 h before collecting the media and use it as a source of the enzyme. As shown in Fig 3.3, the absorbance obtained from treatment was similar to the absorbance from background which means that HepG2 did not secreted any PON1 and the colour was attributed to the autohydrolysis. The reason could be that the concentration of secreted PON1 is very low. Therefore, the cell number was increased (two times) and the volume of the media was also increased in the reaction mixture (9 times). In this experiment, 100 μM 2-HQ was used to calculate PON-1-derived activity. As shown in Fig 3.4, the secreted arylesterase activity increased, although the activity was not derived from PON1 as the activity in the presence and the absence of PON1 inhibitor was identical which indicates that HepG2 did not secrete PON1. Probably, HepG2 synthesised the enzyme endogenously but could not secret it as FBS-free media was used in the treatment so there was not any sources of HDL for the enzyme to be structured on. To test the possibility of the endogenous production of PON1, cell-based PON1 activity was measured.

3.5.1.2. Cell-based PON1

Cell-based PON1 is usually measured by removing the medium after treatment followed by incubation with substrate (reaction mixture) for a certain time prior to measuring change in absorbance.

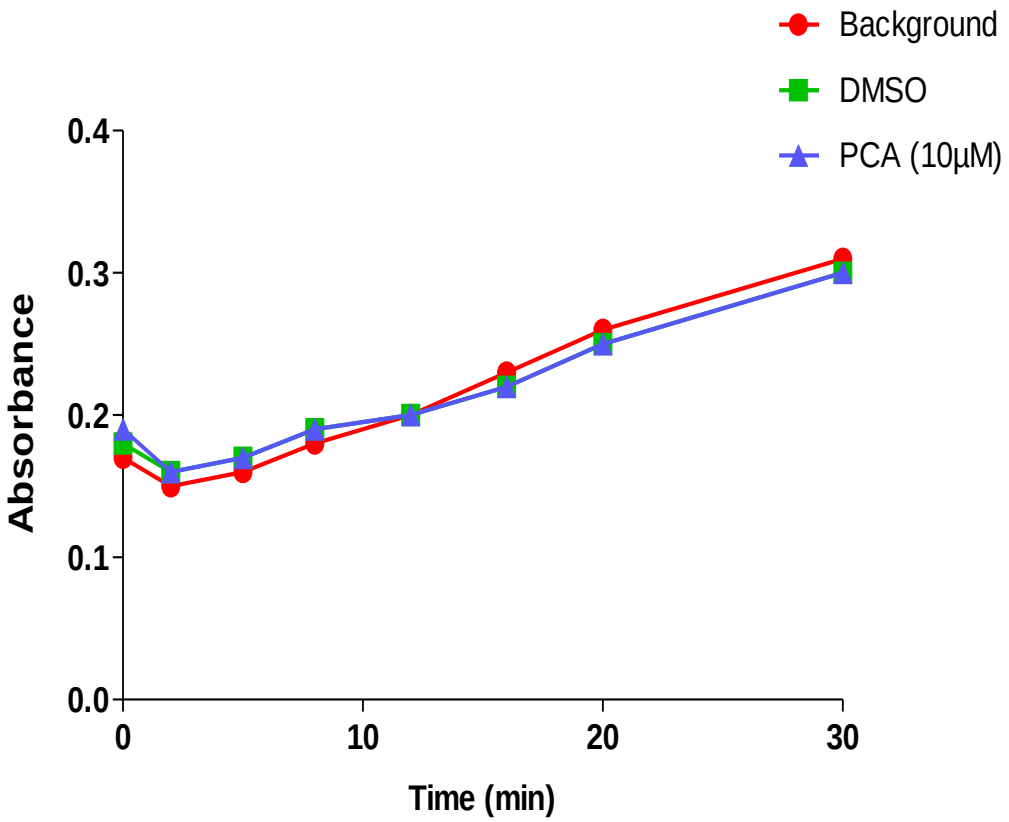


Figure 3. 3: Secreted PON1 arylesterase activity using HepG2 cell.

HepG2 cells ($5x10^5$) were seeded in 6-well plate and treated with treatments for 48 hr. 10 µl of media after treatment was used as a source of PON1. The arylesterase activity was measured using *p*-nitrophenyl acetate (1 mM) as a substrate and monitoring the change in the absorbance at 410 nm. PCA = protocatechuic acid (10 µM). Background was media collected from cell-free well.

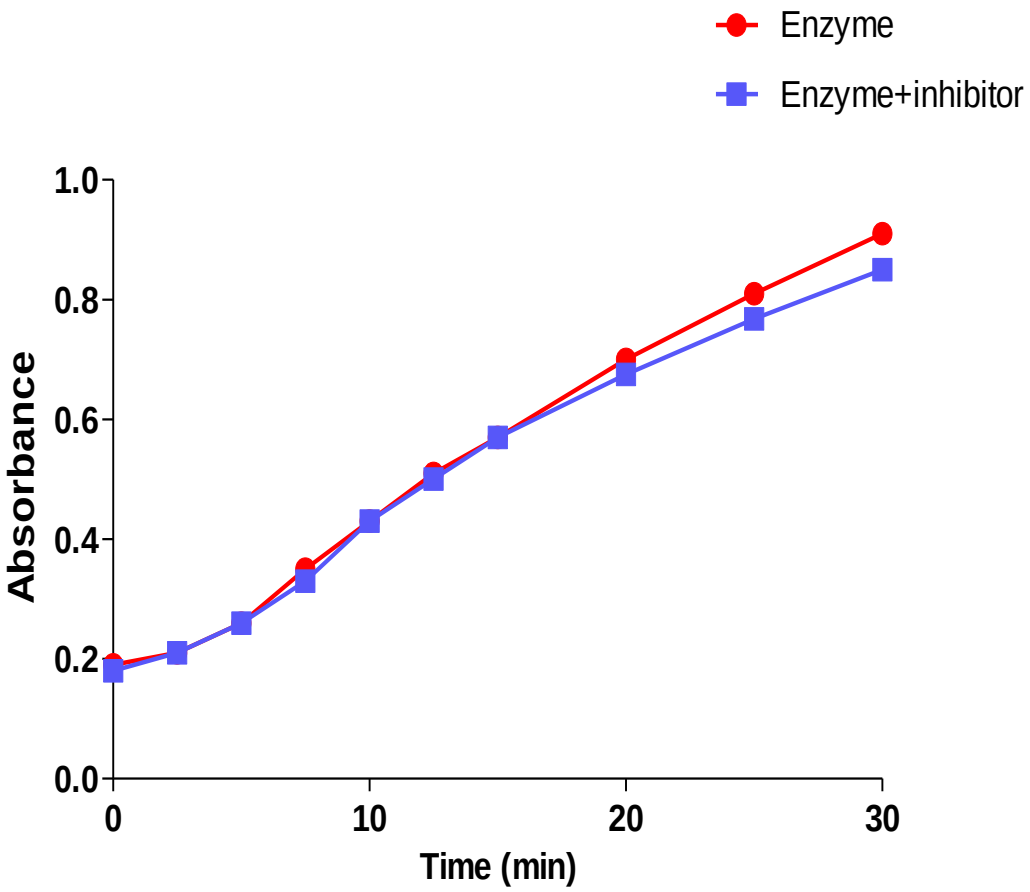


Figure 3. 4: Secreted PON1 arylesterase activity using HepG2 cell.

HepG2 cells ($1x10^6$) were seeded in 6-well plate and treated with DMAO for 48 hr. 90 µl of media after treatment was used as a source of PON1. The arylesterase activity was measured using *p*-nitrophenyl acetate (1 mM) as a substrate with the presence or the absence of 100 µM of 2-HQ and monitoring the change in the absorbance at 410 nm. The measurement was corrected for background which was media collected from cell-free well.

To measure the cell-based PON1 activity, the cells were treated with DMSO and two concentration of aspirin (0.25 and 0.5 mM) which was previously reported to induce cell-associated PON1 by 3-fold [283]. The assay buffer that contain the substrate was added directly to the cell layer following the treatments and after removing the media and washing the cell layer. The assay was performed in presence and absence of 2-HQ, the inhibitor of PON1, to measure the PON1-derived activity.

As shown in Fig 3.5, it was obvious that the total endogenous cell-based arylesterase was higher than the secreted enzyme. The absorbance of cell based arylesterases was about 1.8 unit (Fig 3.5), while, the secreted arylesterases was less than 0.9 unit (Fig 3.4) confirming that the arylesterases were mostly stored in the cells without secretion. However, the data also showed that the cell-based arylesterases activity was not mediated by PON1 as the absorbance in presence and the absence of PON1 inhibitor was similar. In addition, none of the aspirin treatments induced PON1 production (Fig 3.5) suggesting that HepG2 cells model was not suitable for investigating the effects of treatments on PON1 enzyme activity.

3.5.1.3. PON1 gene expression in HepG2

Since, no secreted or cell-based PON1 activity was detected, the expression of PON1 gene was quantified to explore whether PON1 was expressed in HepG2 cells or not. As shown in Fig 3.6, mRNA transcription level of PON1 was very low with Ct values higher than 30 indicating that PON1 gene is lowly expressed in HepG2.

Overall, the HepG2 cells did not synthesize PON1 enzyme and the gene expression was very low suggesting that the used HepG2 cells were not suitable for testing the effects of anthocyanins and/or their metabolites on PON1 activities and gene expression. Instead, a stably transfected cell line with PON1 promoter was used as a model and the promoter activity was measured using the reporter gene assay.

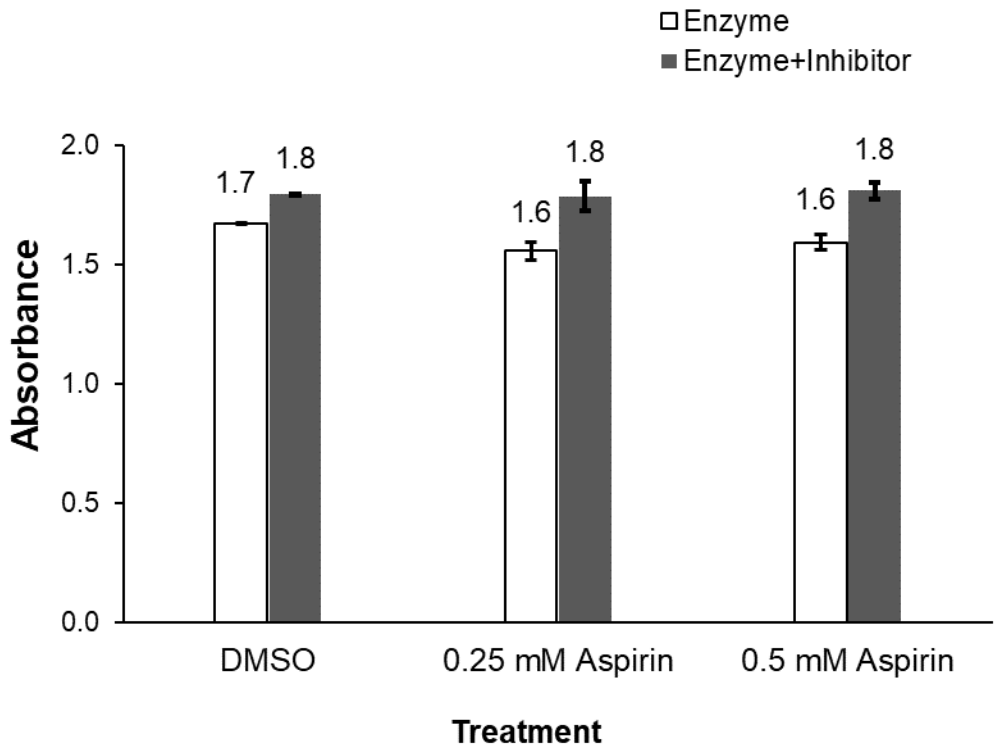


Figure 3. 5: Cell-based PON1 arylesterase activity using HepG2 cell.

HepG2 cells ($5x10^5$) were seeded in 6-well plate and treated with aspirin for 48 hr. The media was then removed, and the cell layer was washed prior to adding the reaction mixture. The cell layer was incubated for 20 min with 1 ml of reaction mixture that contain 1 mM *p*-nitrophenyl acetate with the presence and the absence of PON1 inhibitor (100 µM of 2-HQ). The measurement was corrected for background which was the absorbance from cell-free well. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test.

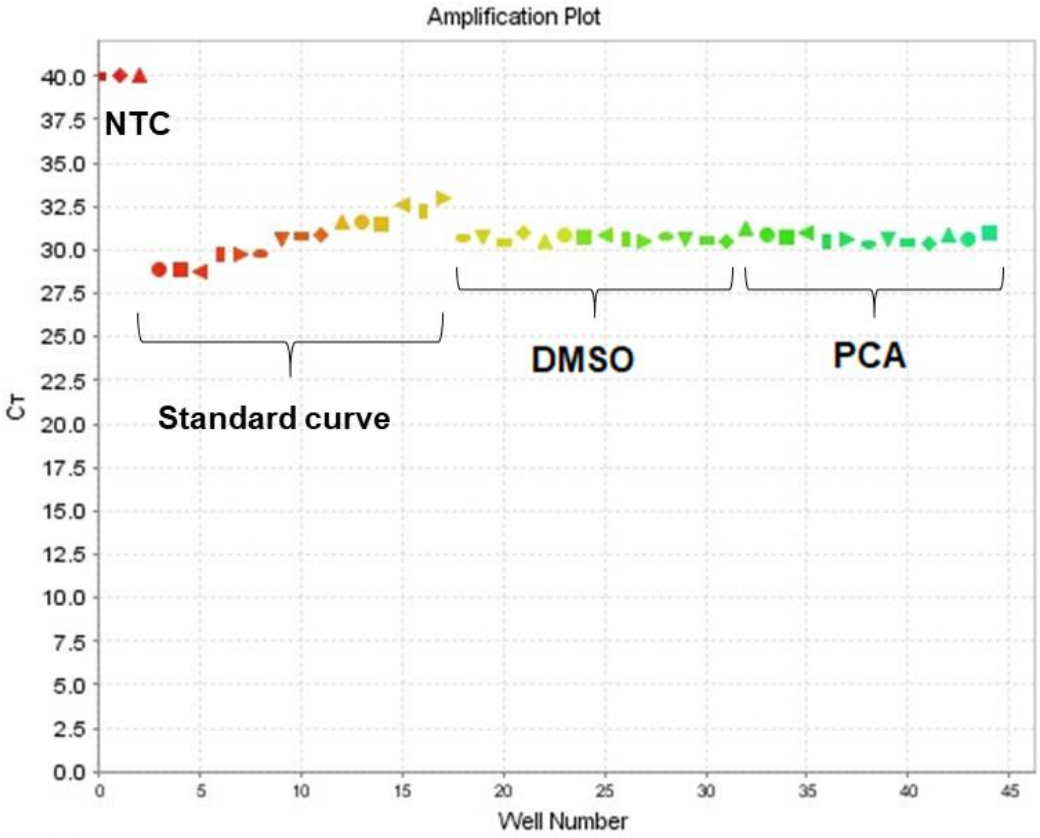


Figure 3. 6: The amplification blot of PON1 gene expression in HepG2.

HepG2 cells were treated with DMSO and 10 µM of PCA = protocatechuic acid for 48 hr and the gene expression was quantified using RT-qPCR method. NTC = no template control, RNA-free water was used as a template instead of RNA samples to monitor contamination. Ct = cycle threshold.

3.5.2. Effect of anthocyanins and their metabolites on PON1 promoter activity.

3.5.2.1. Anthocyanins parent compounds

To test whether the parent anthocyanins affect PON1 gene expression, PON1-Huh7 cells which were stably transfected with PON1 promoter were incubated with C3G and D3G at two concentrations, 1 and 10 μM, for 24 hr at 37°C. 1 μM is physiologically achievable and 10 μM is supra-physiological concentration. PON1 promoter activity was then measured using reporter gene assay. As shown in Fig 3.7, C3G and D3G did not cause any significant change in PON1 promoter activity compared to control (DMSO), although the two concentrations of C3G caused a small increase in promoter activity but this was not significant ($p \geq 0.05$).

In the current model, curcumin was used as a positive control which significantly increased PON1 promoter activity by 5.3-fold (Fig 3.7, $p \leq 0.001$)). This was consistent with data previously reported that curcumin induced PON1 promoter activity by 4.5-fold. This demonstrates the current model is functioning properly and it is sensitive to treatments [282], [290].

3.5.2.2. Anthocyanins metabolites and their conjugates

To test the hypothesis that anthocyanin metabolites induce PON1 promoter activity, the anthocyanin metabolites used in the previous chapter were applied to PON1-Huh7 cells for 24 hr at 1 and 10 μM. Fig 3.8 reports the effects of known metabolites of anthocyanins that were identified previously by using isotope-labelled anthocyanins, while, Fig 3.9 reports the effects of other predicted metabolites of anthocyanins. Similar to the parent compounds, none of these metabolites significantly changed PON1 promoter activity at either concentration (Fig 3.8 and 3.9). However, syringic, cyanidin, 5HFA and hippuric acid unexpectedly reduced the promoter activity by 10 to 20%, although the changes were not significant (Fig 3.9).

Moreover, PCA conjugates and gallic acid conjugates did not induce the promoter activity (Fig 3.10). Likewise, no significant changes were seen with a combination of C3G, PCA and its conjugates or with a combination of D3G, gallic acid and its conjugates (Fig 3.10). Although, there was a small decrease in PON1 promoter activity with the tested conjugates and mixtures except for a mixture of C3G, PCA and its conjugates (Fig 3.10), this was not significant ($p \geq 0.05$).

Curcumin (the positive control), on the other hand, significantly increased PON1 promoter activity by 5.3-fold (Fig 3.8 - 3.10) which indicates that the model is working and confirms that the lack of effect of the tested metabolites of anthocyanins on PON1 promoter activity and consequently PON1 gene expression were real. However, anthocyanins and their metabolites may target PON1 at different levels such as increasing the enzyme activity. Therefore, the effect of anthocyanins and their metabolites on PON1 enzyme activities were investigated.

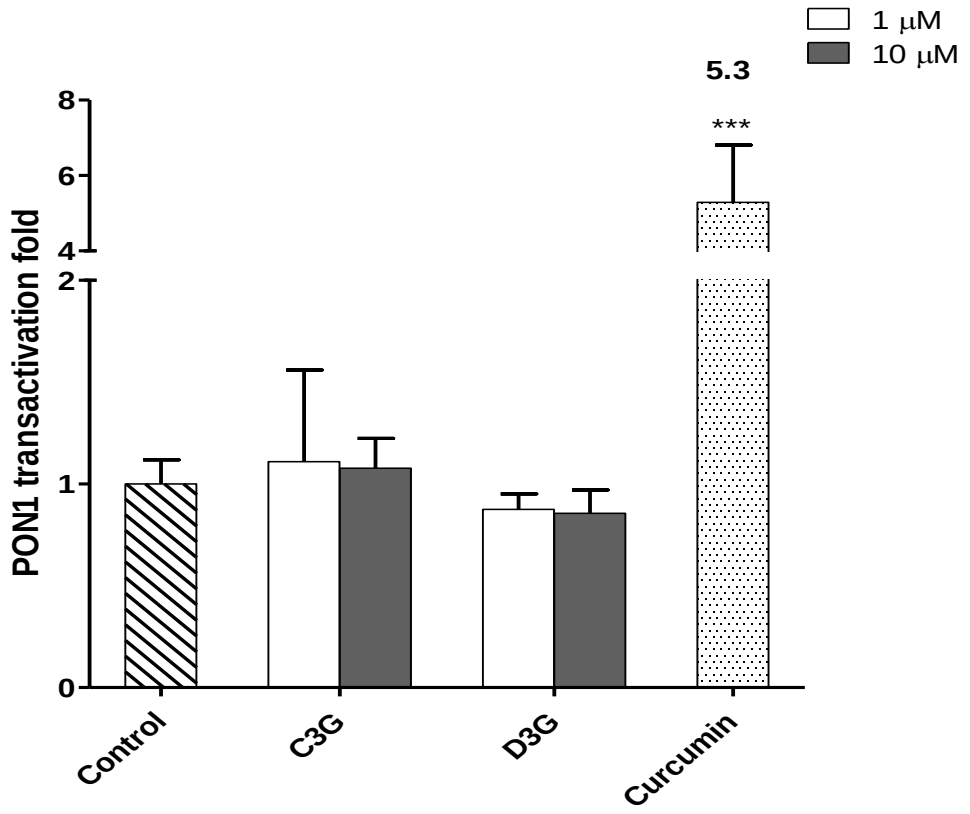


Figure 3. 7: Effect of anthocyanins parent compounds on PON1 promoter activity.

PON1-Huh7 cells were treated with treatments for 24 hr followed by protein extraction and measuring the promoter activity using the reporter gene assay. Control = DMSO, C3G = cyanidin-3-glucoside, D3G = delphinidin-3-glucoside and curcumin (20 μM) was the positive control. The luminescence of the reporter was normalized to the protein content. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in quadruplicates and the experiments were repeated two times.

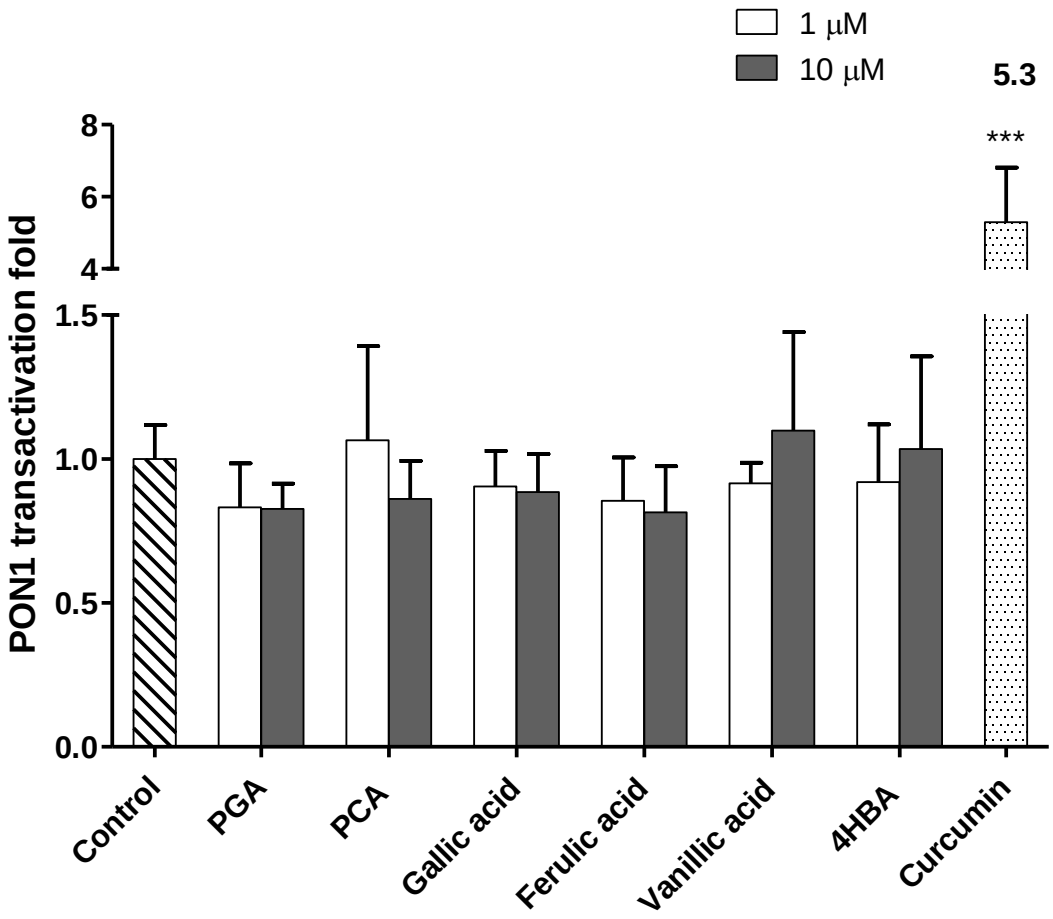


Figure 3. 8: Effect of known human anthocyanins metabolites on PON1 promoter activity.

PON1-Huh7 cells were treated for 24 hr followed by protein extraction and measurement of the promoter activity using the reporter gene assay. Control = DMSO, PGA = phloroglucinaldehyde, PCA = protocatechuic acid, 4HBA = 4-hydroxybenzaldehyde and curcumin (20 μM) was the positive control. The luminescence of the reporter was normalized to the protein content. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in quadruplicates and the experiments were repeated two times.

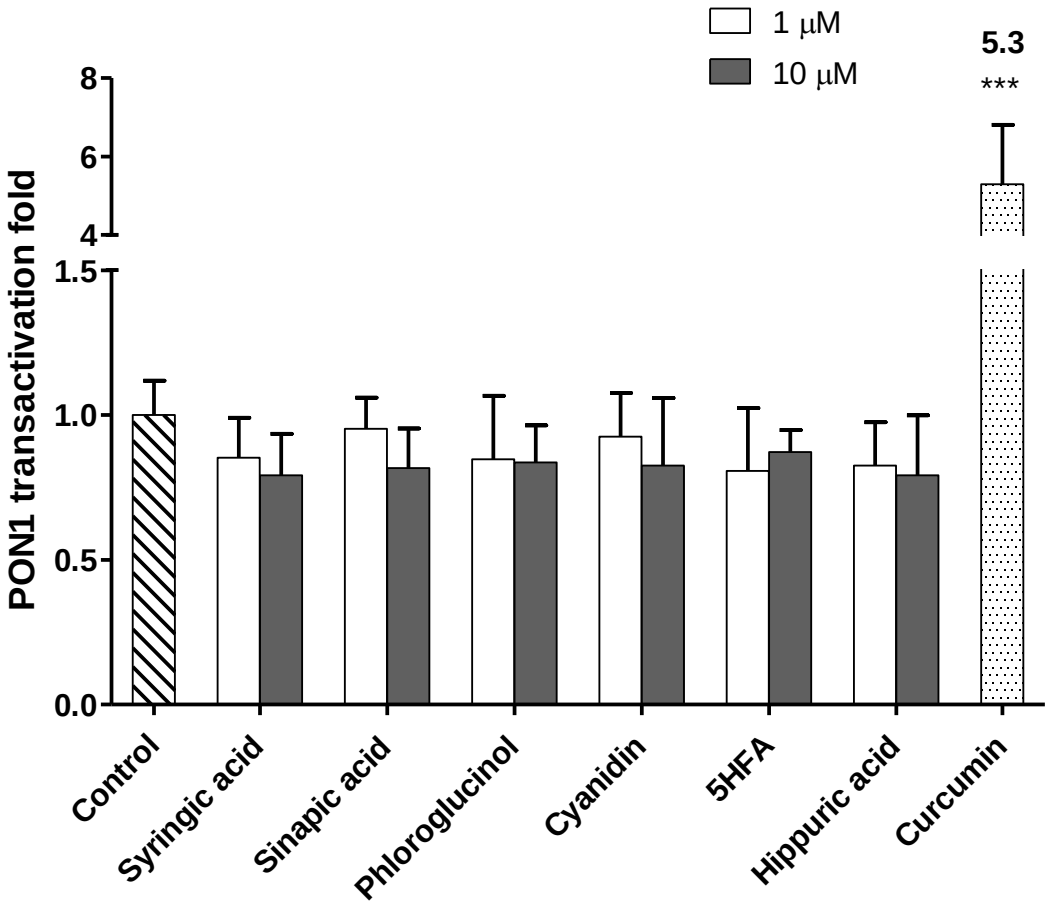


Figure 3. 9: Effect of potential anthocyanins metabolites on PON1 promoter activity.

PON1-Huh7 cells were treated for 24 hr followed by protein extraction and measurement of the promoter activity using the reporter gene assay. Control = DMSO, 5HFA = 5-hydroxyferulic acid and curcumin (20 μM) was the positive control. The luminescence of the reporter was normalized to the protein content. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in quadruplicates and the experiments were repeated two times.

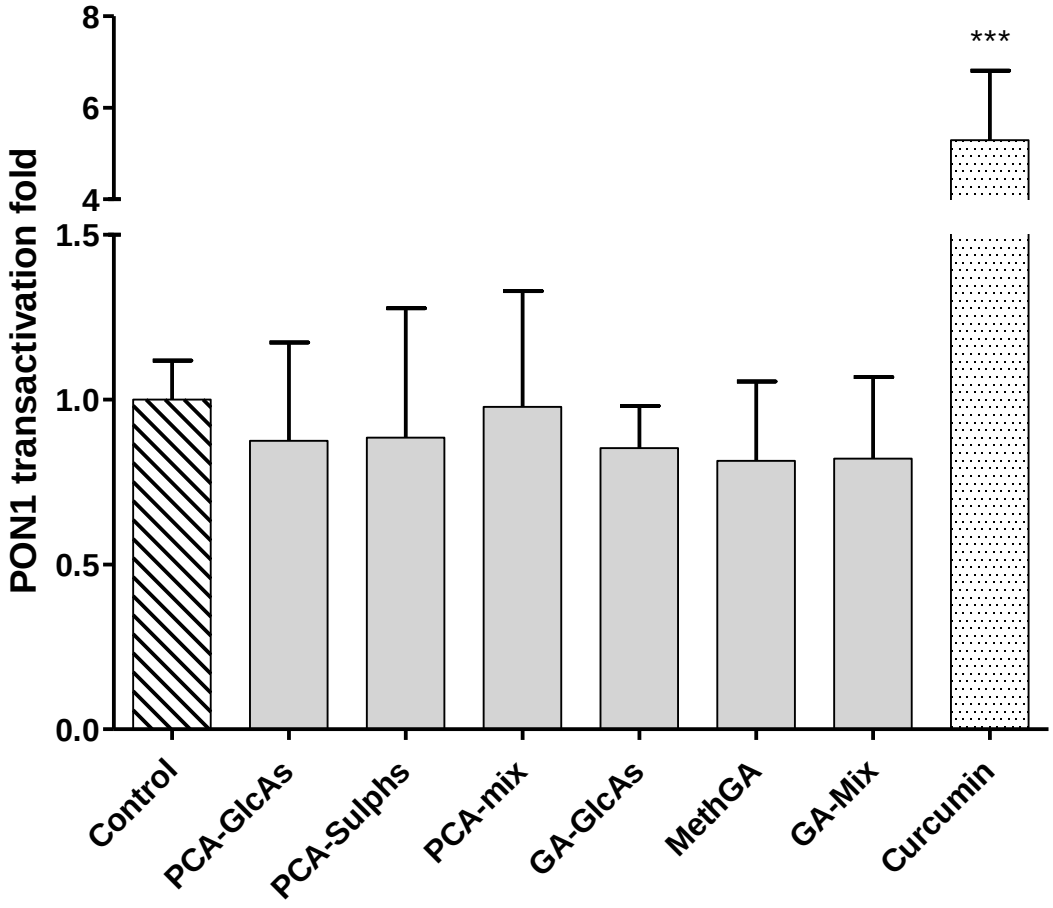


Figure 3. 10: Effect of phase-ii-conjugates of anthocyanin metabolites on PON1 promoter activity.

PON1-Huh7 cells were treated with treatments for 24 hr followed by protein extraction and measurement of the promoter activity using the reporter gene assay. Control = DMSO, PCA-GlcAs (1 µM of PCA-3-glucuronide and PCA-4-glucuronide), PCA-Sulphs (1 µM of PCA-3-sulphate and PCA-4-sulphate), PCA-Mixture (1 µM of C3G, PCA and PCA conjugates), GA-GlcAs (1 µM of gallic acid-3-glucuronide and gallic acid-4-glucuronide), MethGA (1 µM of 3-*O*- Methylgallic acid and 4-*O*- Methylgallic acid), GA-Mixture (1 µM of D3G, gallic acid and gallic acid conjugates) and curcumin (20 µM) was the positive control. The luminescence of the reporter was normalized to the protein content. The fold change was calculated relative to control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in quadruplicates and the experiments were repeated two times.

3.5.3. Effect of anthocyanins and their metabolites on PON1 arylesterase activity.

3.5.3.1. Establishing a fit-for-purpose enzyme assay

The enzyme assay is simply measured by monitoring the generation of the product of the enzyme reaction over time using a spectrophotometer. One advantage of measuring the absorbance over time rather than the endpoint is to avoid interference from other coloured compounds otherwise the product of the enzyme reaction. If the interfering compounds are inactive, their colour will remain unchanged over time and therefore, the rate will represent only the enzyme activity. If the enzyme activity was measured in an endpoint assay, the interfering compounds would be detected as a false positive if their absorbance started at a high value and no correction was done. To avoid inaccurate measurement, the rate should be measured within the linear stage of the enzyme reaction. Therefore, serial dilutions of PON-RR and PON-QQ enzymes in buffer were tested. The rate of hydrolysis of p-nitrophenyl acetate, the substrate, was measured using a colourimetric method.

A concentration of 1% PON-RR (0.63 U/ml) and 0.7% PON-QQ (1.3 U/ml) enzymes resulted in a linear reaction which lasted for more than 15 min (Fig 3.11). The linear regression coefficient (R^2) was 0.9997 which is very close to 1 meaning that the reaction is linear at these two concentrations of PON-RR and PON-QQ. As shown in Fig 3.11, the linearity lasted for 16 min or more which gave enough time to measure the plate after adding the substrate. Therefore, the rate was measured over 10 min using these two concentrations in all subsequent experiments.

To validate that the commercial enzyme does not contain any other esterases rather than PON1 and to make sure that the model was working, 2-hydroxyquinoline (2-HQ), a potent inhibitor of PON1, was used at concentration of 100 µM. 2-HQ resulted in about 97.5% inhibition for PON-RR and PON-QQ which mean that the product contains only PON1 and the model is working properly (Fig 3.11).

3.5.3.2. Effect of anthocyanins parent compounds on PON1 arylesterase activity.

In this study, the direct effect of anthocyanin parent compounds on PON1 arylesterase activity was examined using two different phenotypes, PON-RR and PON-QQ. The two enzymes were incubated with C3G and D3G at concentrations of 1 and 10 µM for 10 min at 37°C. The substrate, p-nitrophenyl acetate, then was added, and the

reaction rate was colourimetrically measured over 10 min. The findings demonstrated that neither C3G or D3G affected the arylesterase activity at either concentration of either the PON-RR or PON-QQ enzyme (Fig 3.12 A&B). These data do not support the notion that anthocyanins interact directly with PON1 enzymes to change their enzyme activity. It is possible that the breakdown products and/or microbial metabolites affect PON1 activity, so this was investigated.

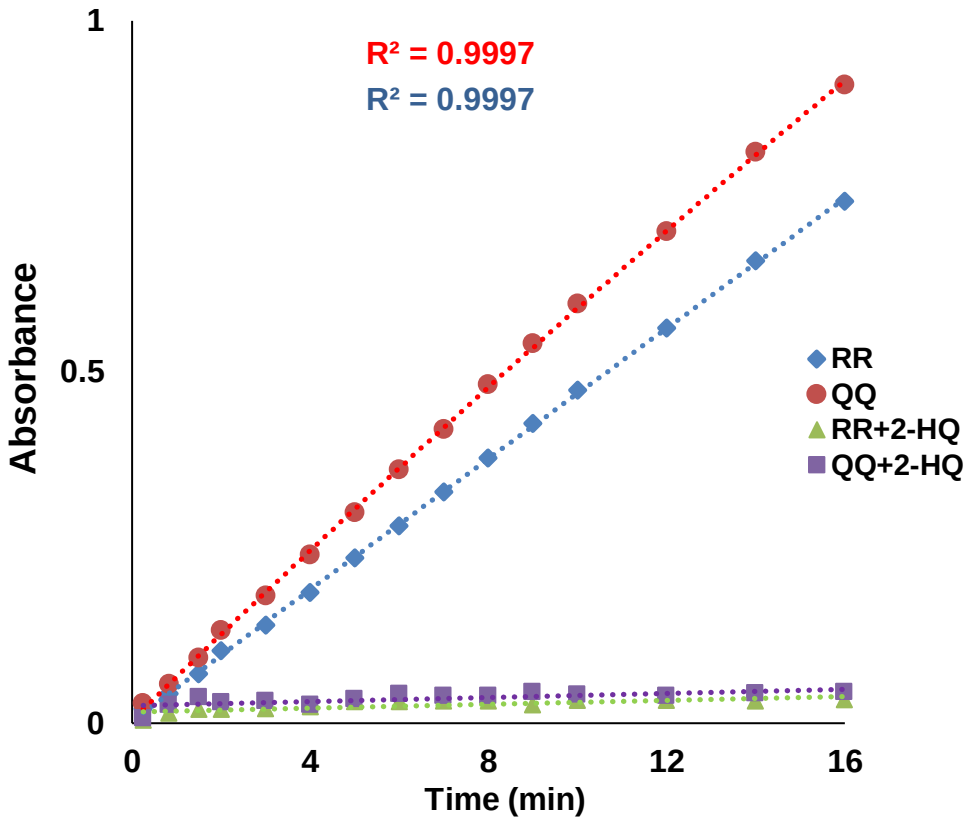


Figure 3. 11: PON1 arylesterase reaction progress curve.

The substrate was added to 1% PON-RR and 0.7% PON-QQ with and without 100 µM 2-hydroxyquinoline. The enzymes were diluted in buffer. The absorbance was recorded over 16 min. R^2= linear regression coefficient. R^2 was calculated using Excel software 2016.

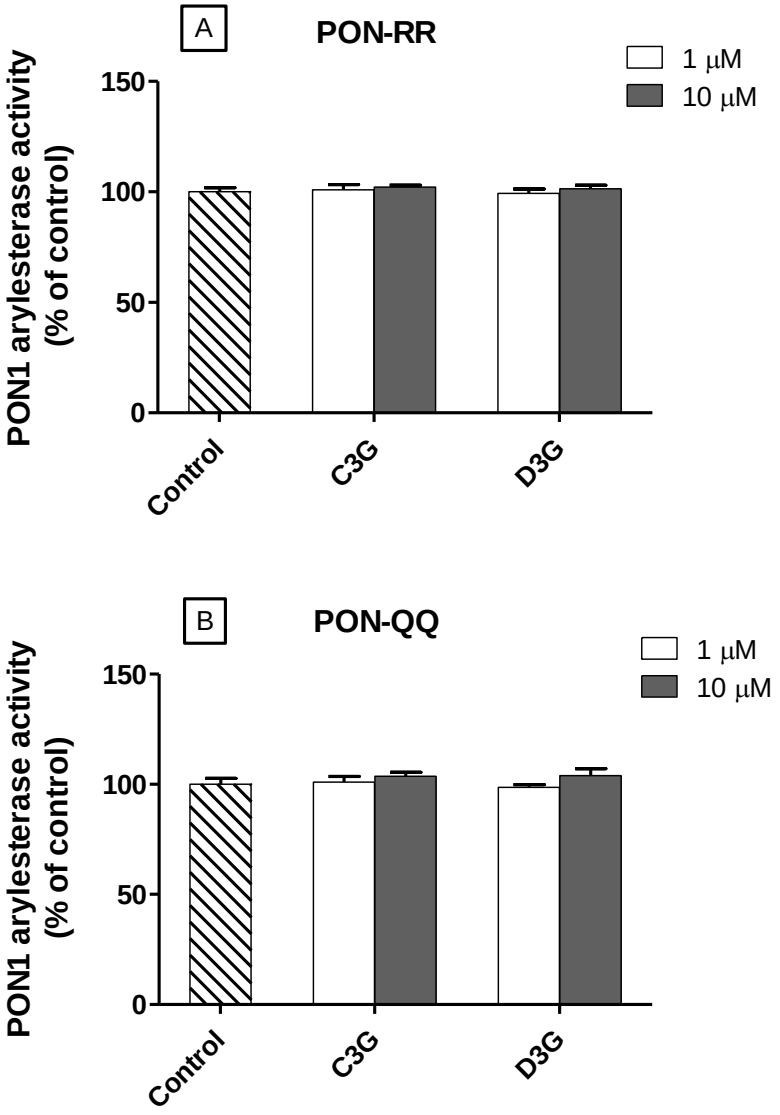


Figure 3. 12: Effect of anthocyanin parent compounds on (A) PON-RR and (B) PON-QQ arylesterase activity.

PON-RR and PON-QQ enzymes were incubated with treatments for 10 min at 37ºC. Control = DMSO (0.1%), C3G = cyanidin-3-glucoside and D3G = delphinidin-3-glucoside for 10 min at 37ºC. The slope of the reaction was calculated using the instrument software. The % of change to the slope was calculated relative to the control. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in triplicates and the experiments were repeated two times.

3.5.3.3. Effect of anthocyanins metabolites on PON1 arylesterase activity

To measure the effect of anthocyanin metabolites on PON1 arylesterase, the same protocol was used. Two types of metabolites were tested. Firstly, metabolites that are known to be or were predicted to be formed by either spontaneous or gut microbiota-dependent breakdown/catabolism of the anthocyanins. In addition, phase-ii-conjugates of the phenolic breakdown products formed as a result of human metabolism were also examined.

The 10 µM gallic acid treatment significantly increased PON-QQ arylesterase but not PON-RR by 9% compared to the control (Fig 3.13 B, $p \leq 0.001$). Similarly, phloroglucinol at both 1 and 10 µM increased PON-QQ but not PON-RR but by only 4% (Fig 3.14 B, $p \leq 0.001$). On the other hand, the high concentration of cyanidin (10 µM) but not the low concentration slightly decreased PON-RR by 6% (Fig 3.14 A, $p \leq 0.001$). Otherwise, none of the other tested metabolites either the confirmed one or the potential caused any change in arylesterase activity with PON-RR or PON-QQ at any tested concentrations (Fig 3.13-3.14).

Next, some phase-ii-conjugates of the metabolites were tested. None of the tested conjugates affected PON1 arylesterase activity (Fig 3.15). It was also shown that neither a mixture of C3G, PCA and its conjugates nor a mixture of D3G, gallic acid and its conjugates caused any significant changes in PON1 activity (Fig 3.15).

Overall, none of the treatments had any substantial effects on PON1 activity. Although statistically significant effects were observed for gallic acid (increased PON-QQ by 9% at 10 µM) and phloroglucinol (increase PON-QQ by 4% at 1 and 10 µM), the effects were small.

PON1 is ‘promiscuous’ multi-activity enzyme. In addition to arylesterase activity, the enzyme possesses lactonase activity (see section 3.2.2). Indeed, lactonase is the enzyme function associated with it physiological function, especially in its action towards ox-LDL. Therefore, the effect of anthocyanins and their metabolites on lactonase activity have been investigated

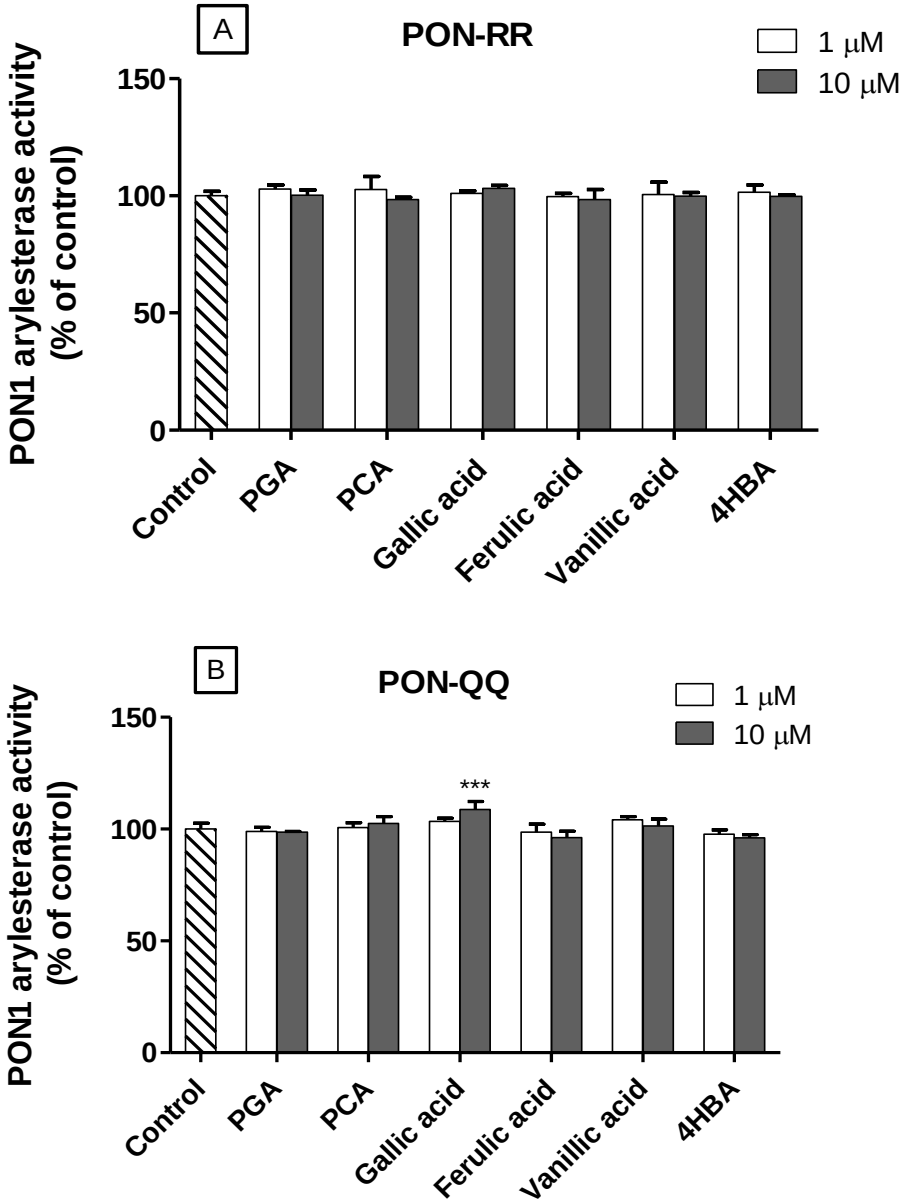


Figure 3. 13: Effect of known human anthocyanin metabolites on (A) PON-RR and (B) PON-QQ arylesterase activity.

PON-RR and PON-QQ enzymes were incubated with treatments for 10 min at 37ºC. Control = DMSO (0.1%), PGA = phloroglucinaldehyde, PCA = protocatechuic acid and 4HBA = 4-hydroxybenzaldehyde. The slope of the reaction rate was calculated using the instrument software. The % of change to the slope was calculated relative to the control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in triplicates and the experiments were repeated two times

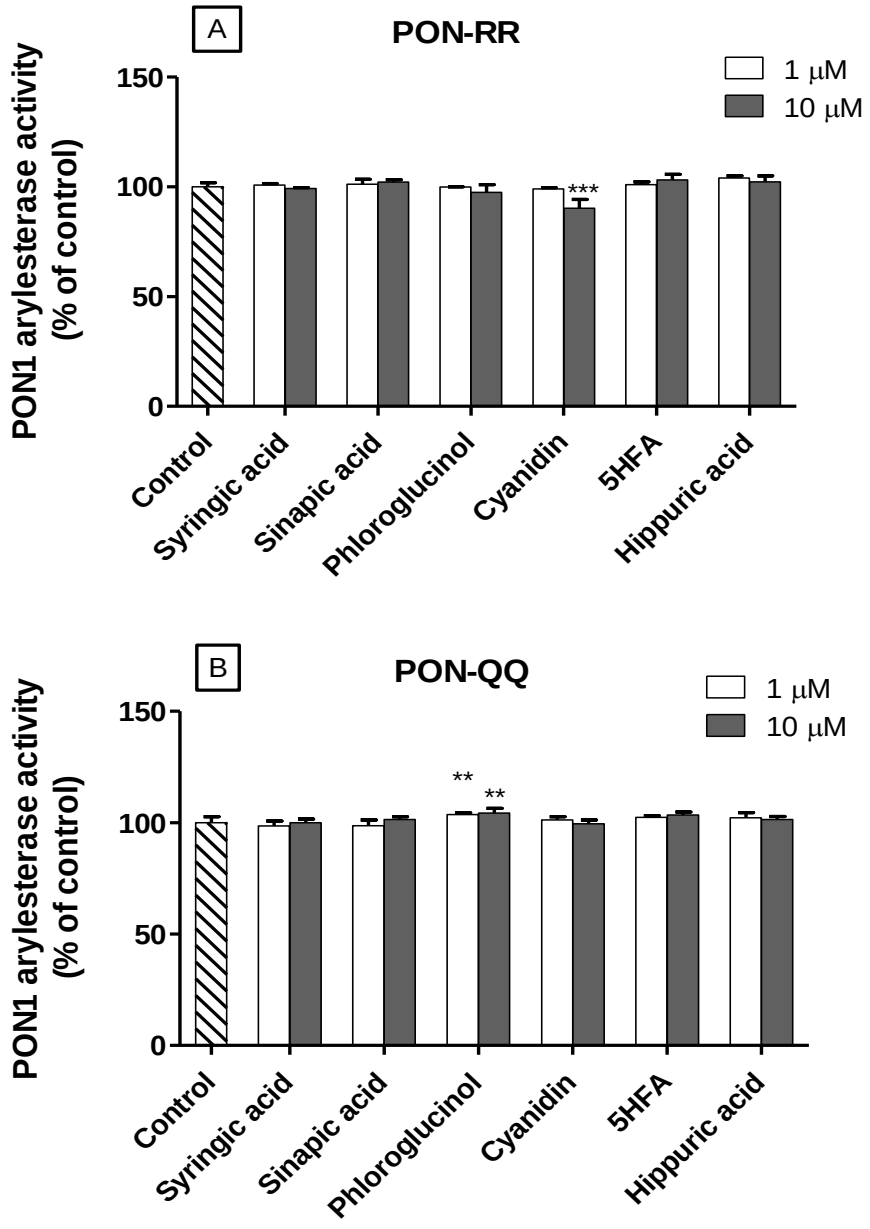


Figure 3. 14: Effect of anthocyanin potential metabolites on (A) PON-RR and (B) PON-QQ arylesterase activity.

PON-RR and PON-QQ enzymes were incubated with treatments for 10 min at 37ºC. Control = DMSO (0.1%), 5HFA = 5-hydroxyferulic acid. The slope of the reaction rate was calculated using the instrument software. The % of change to the slope was calculated relative to the control. Data are shown as means ± SD. ** $p \leq 0.01$ and *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in triplicates and the experiments were repeated two times.

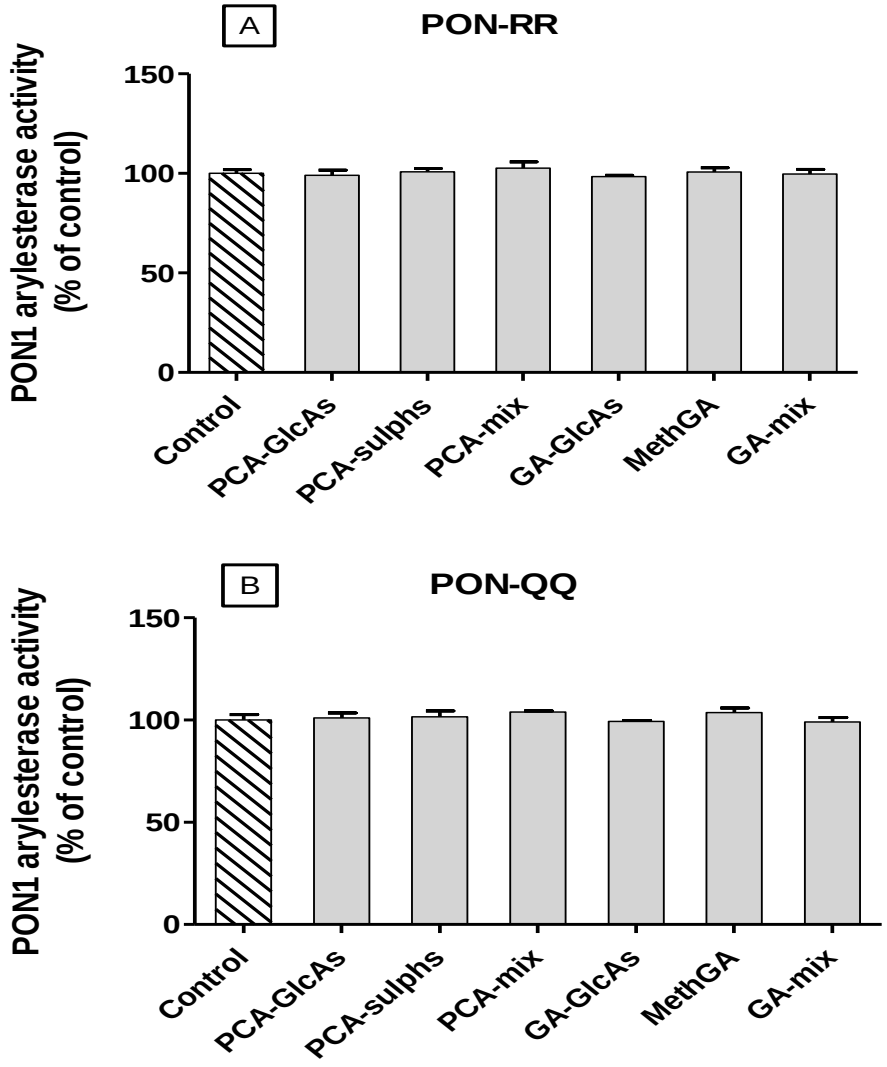


Figure 3. 15: Effect of anthocyanin phase-ii-metabolite conjugates on (A) PON-RR and (B) PON-QQ arylesterase activity.

PON-RR and PON-QQ enzymes were incubated with treatments for 10 min at 37ºC. Control = DMSO, PCA-GlcAs = (1 µM of PCA-3-glucuronide and PCA-4-glucuronide), PCA-Sulphs = (1 µM of PCA-3-sulphate and PCA-4-sulphate), PCA-Mixture = (1 µM of C3G, PCA and PCA conjugates), GA-GlcAs = (1 µM of gallic acid-3-glucuronide and gallic acid-4-glucuronide), MethGA = (1 µM of 3-*O*- methylgallic acid and 4-*O*- methylgallic acid), GA-Mixture = (1 µM of D3G, gallic acid and gallic acid conjugates). The slope of the reaction rate was calculated using the instrument software. The % of change to the slope was calculated relative to the control. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in triplicates and the experiments were repeated two times.

3.5.4. Effect of anthocyanins and their metabolites on PON1 lactonase activity.

3.5.4.1. Setting up the assay

Similar to the arylesterase activity, a serious of enzyme dilutions were performed to obtain a linear lactonase activity to allow accurate measurement. The rate of the cleavage of TBBL, the substrate of lactonase activity, was measured by monitoring the absorbance over time. Linearity was achieved with 0.2% PON-RR (0.13 U/ml) and with 0.125% PON-QQ (0.23 U/ml); and for both isoforms lasted for more than 15 min. The linear regression coefficient (R^2) was about 0.996 for both phenotypes (Fig 3.16) meaning that the enzyme reaction rate was linear. Moreover, 2-HQ inhibited PON-RR and PON-QQ by 95 and 93%, respectively meaning that the observed changes in absorbance were due to PON1 lactonase activity and not an artefact, and the model is working well (Fig 3.16).

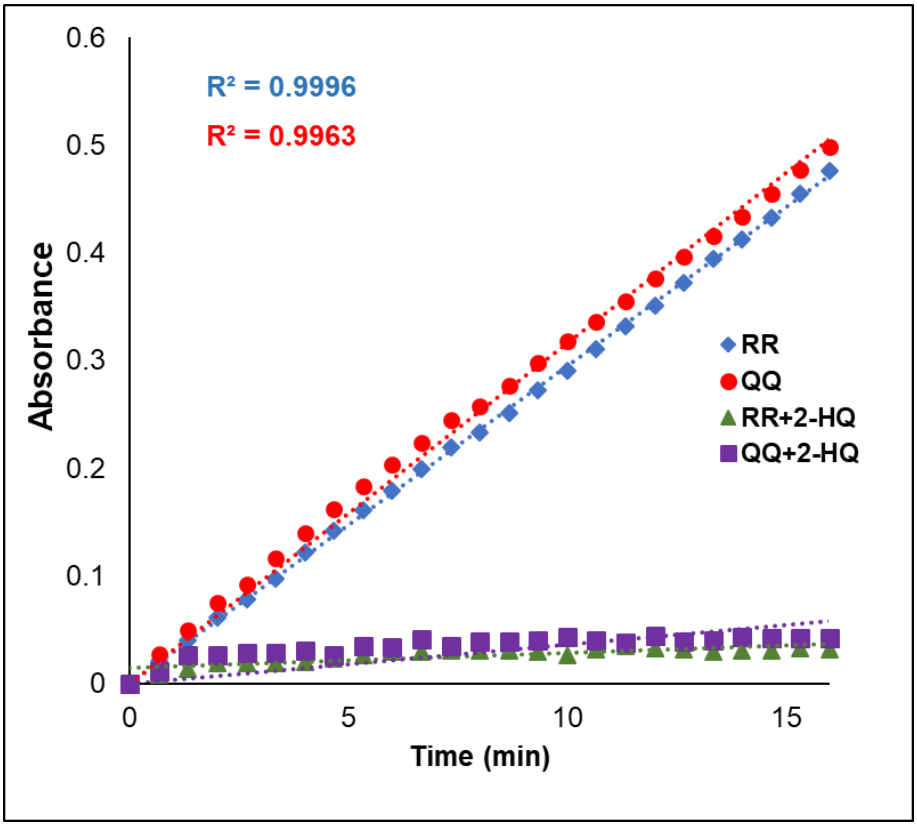


Figure 3. 16: PON1 lactonase reaction progress curve.

The substrate was added to 0.2% PON-RR and 0.125% PON-QQ with and without 100 µM 2-hydroxyquinoline. The enzymes were diluted in buffer. The absorbance was recorded over 16 min. R^2= linear regression coefficient. R^2 was calculated using Excel software 2016.

3.5.4.2. Effect of anthocyanins parent compounds on PON1 lactonase activity.

C3G and D3G at concentrations of 1 and 10 µM were incubated with PON-RR and PON-QQ for 10 min at 37°C. After adding the substrate, TBBL, the lactonase activity was measured over 10 min and the reaction rate was measured to calculate the % change. As shown in Fig 3.17, C3G had a very small but significant effect on the enzyme activity of both PON-RR and PON-QQ. PON-QQ activity was affected more than PON-RR with increase of 11 and 9% at concentrations of 1 and 10 µM, respectively ($p \leq 0.001$). On the other hand, D3G did not cause any changes in PON-RR, while the 1 µM concentration modestly increased PON-QQ (5%, $p \leq 0.001$) (Fig 3.17).

3.5.4.3. Effect of anthocyanins metabolites on PON1 lactonase activity

To investigate the effect of anthocyanin metabolites on PON1 lactonase activity, the same metabolites of anthocyanins that were tested in the arylesterase were tested for their effect on PON1 lactonase activity. A few of the tested metabolites caused very small changes in PON1 lactonase activity, although they were statistically significant (Fig 3.18 and 3.19). The only substantial effect was observed with cyanidin (Fig 3.19 A). Unexpectedly, cyanidin at 10 µM significantly decreased PON-RR lactonase activity by 22% ($p \leq 0.001$), while, the lower concentration of cyanidin did not cause any changes (Fig 3.19 A). This finding was confirmed by doing a dose-response curve for cyanidin with PON-RR enzyme. As shown in Fig 3.20, cyanidin decreased lactonase activity of PON-RR in a dose-dependent manner with about 30% reduction when incubated with 20 µM cyanidin. However, the lower more physiological concentrations of cyanidin (0.1 or 0.5 µM) did not affect PON-RR nor did 1 µM. On the other hand, PON-QQ behaved differently when treated with cyanidin. The low concentration of cyanidin (1 µM) but not the high one slightly increased PON-QQ by 8% (Fig 3.19 B, $p \leq 0.001$).Regarding the effect of anthocyanin metabolite conjugates and the mixtures, no substantial effects were observed with either PON-RR or PON-QQ (Fig 3.21 A &B). Only a very small decrease in PON-RR and PON-QQ was observed after treating with MethGA ($p \leq 0.001$).

In summary, the results showed that none of the tested biomarkers of the RCT pathway were affected by anthocyanins or their metabolites *in vitro*. However, other CVD-related pathways may be targeted. To investigate this further, RNA sequencing has been used as a non-targeted approach to define any other affected CVD-related pathways.

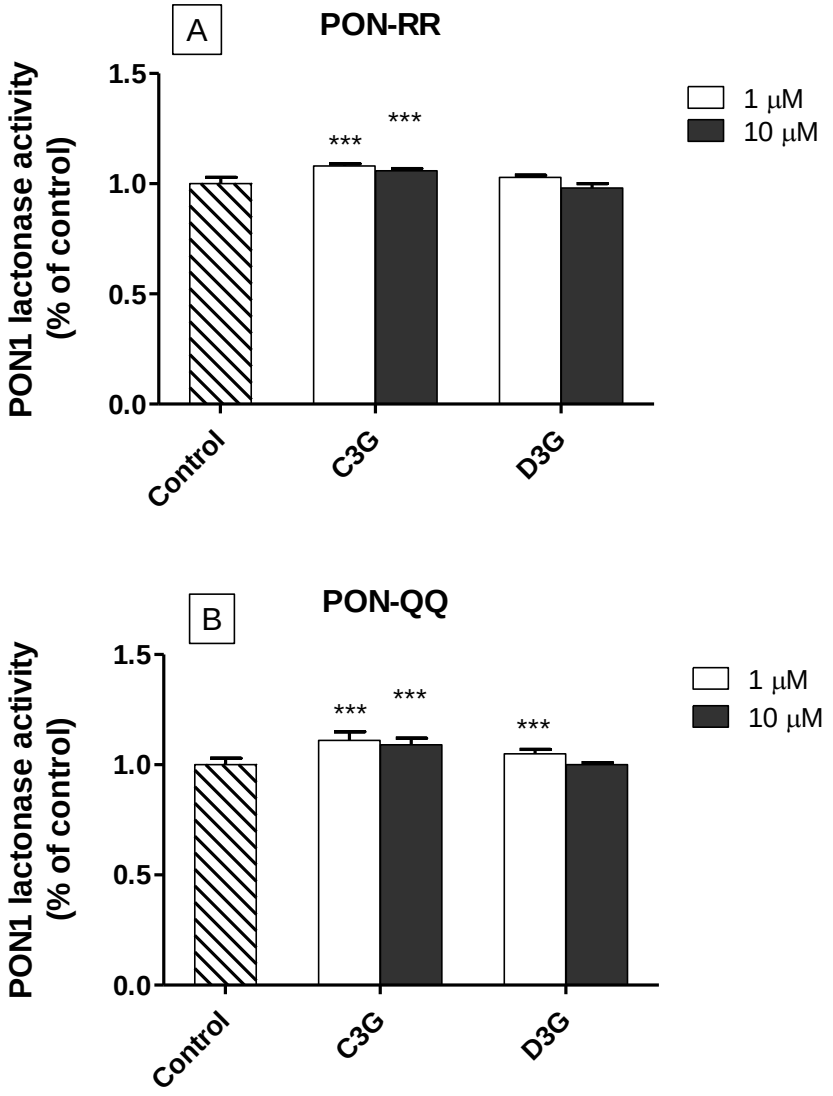


Figure 3. 17: Effect of anthocyanin parent compounds on (A) PON-RR and (B) PON-QQ lactonase activity.

PON-RR and PON-QQ enzymes were incubated with treatments for 10 min at 37ºC. Control = DMSO (0.1%), C3G = cyanidin-3-glucoside and D3G = delphinidin-3-glucoside. The slope of the reaction rate was calculated using the instrument software. The % of change to the slope was calculated relative to the control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in quadruplicates and the experiments were repeated at least two times.

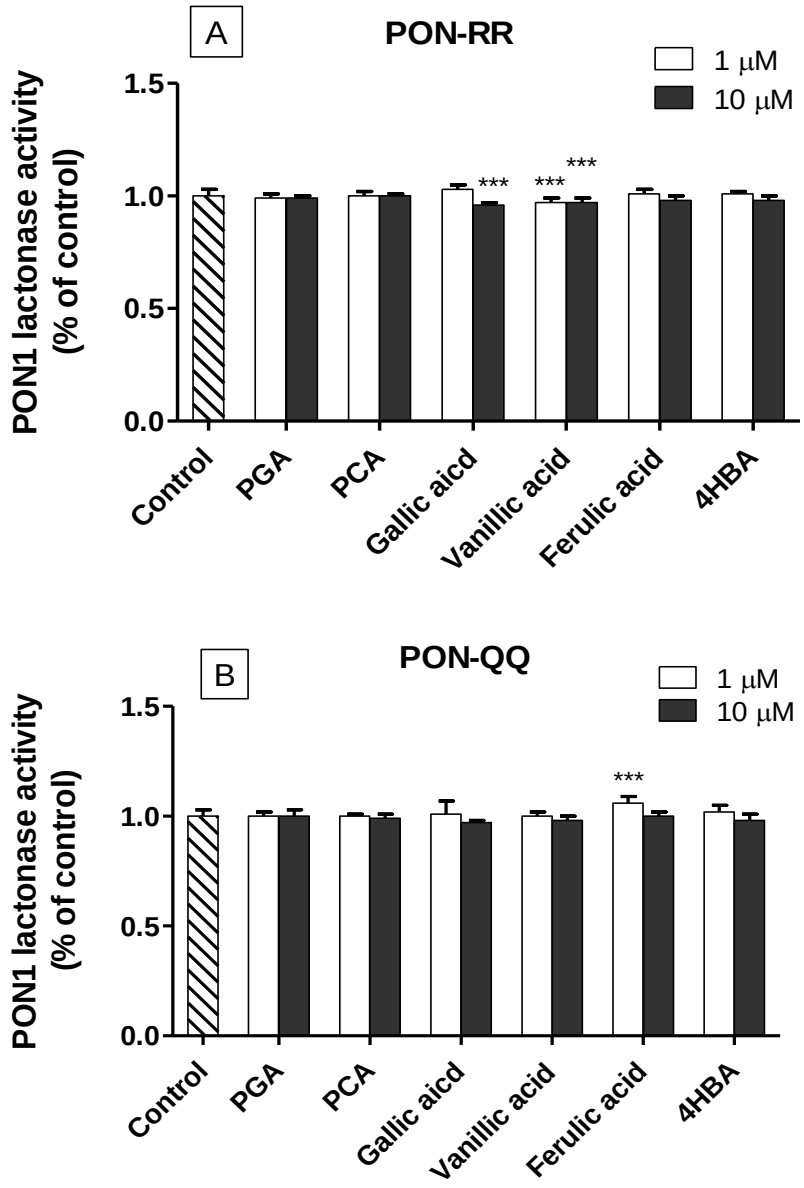


Figure 3. 18: Effect of known human anthocyanin metabolites on (A) PON-RR and (B) PON-QQ lactonase activity.

PON-RR and PON-QQ enzymes were incubated with treatments for 10 min at 37ºC. Control = DMSO (0.1%), PGA = phloroglucinaldehyde, PCA = protocatechuic acid and 4HBA = 4-hydroxybenzaldehyde. The slope of the reaction rate was calculated using the instrument software. The % of change to slope was calculated relative to the control. Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in quadruplicates and the experiments were repeated at least two times.

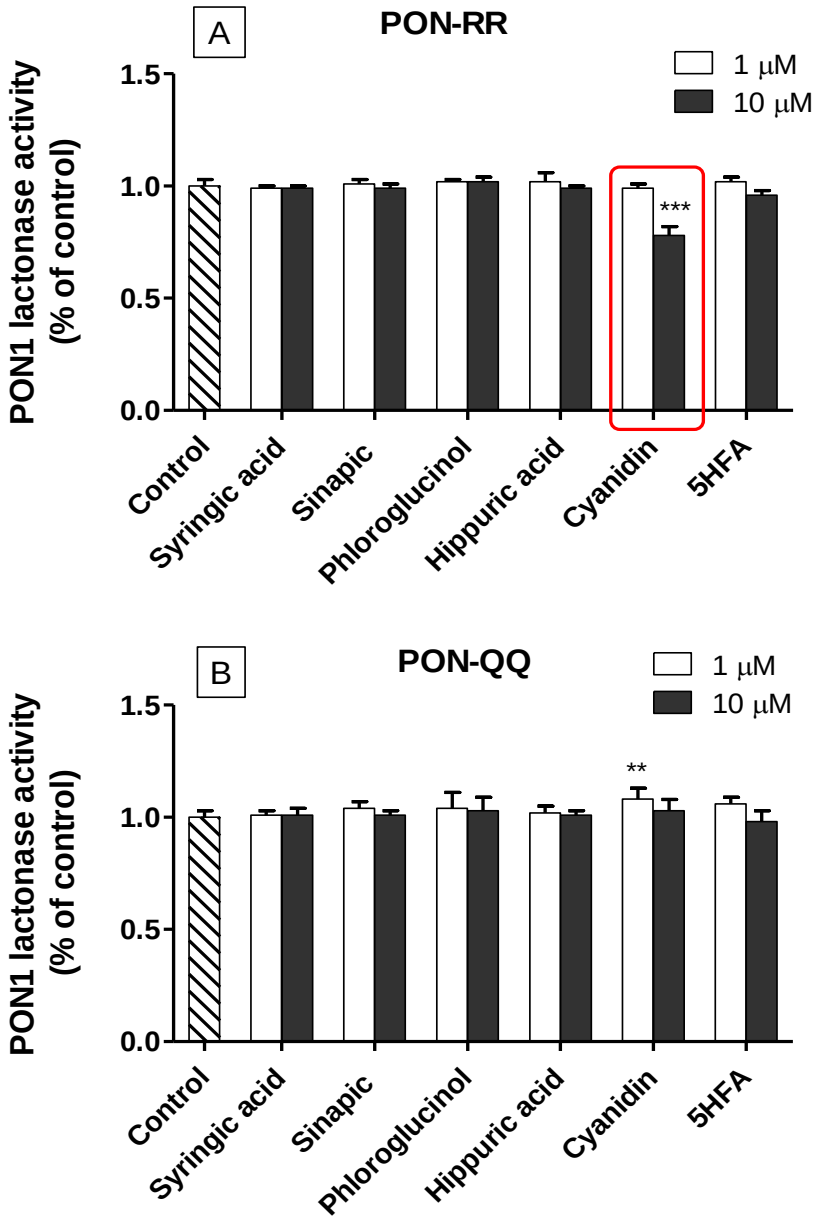


Figure 3. 19: Effect of putative anthocyanin metabolites on (A) PON-RR and (B) PON-QQ lactonase activity.

PON-RR and PON-QQ enzymes were incubated with treatments for 10 min at 37ºC. Control = DMSO (0.1%), 5HFA = 5-hydroxyferulic acid. The slope of the reaction rate was calculated using the instrument software. The % of change to the slope was calculated relative to the control. Data are shown as means ± SD. ** $p \leq 0.01$ and *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in quadruplicates and the experiments were repeated at least two times.

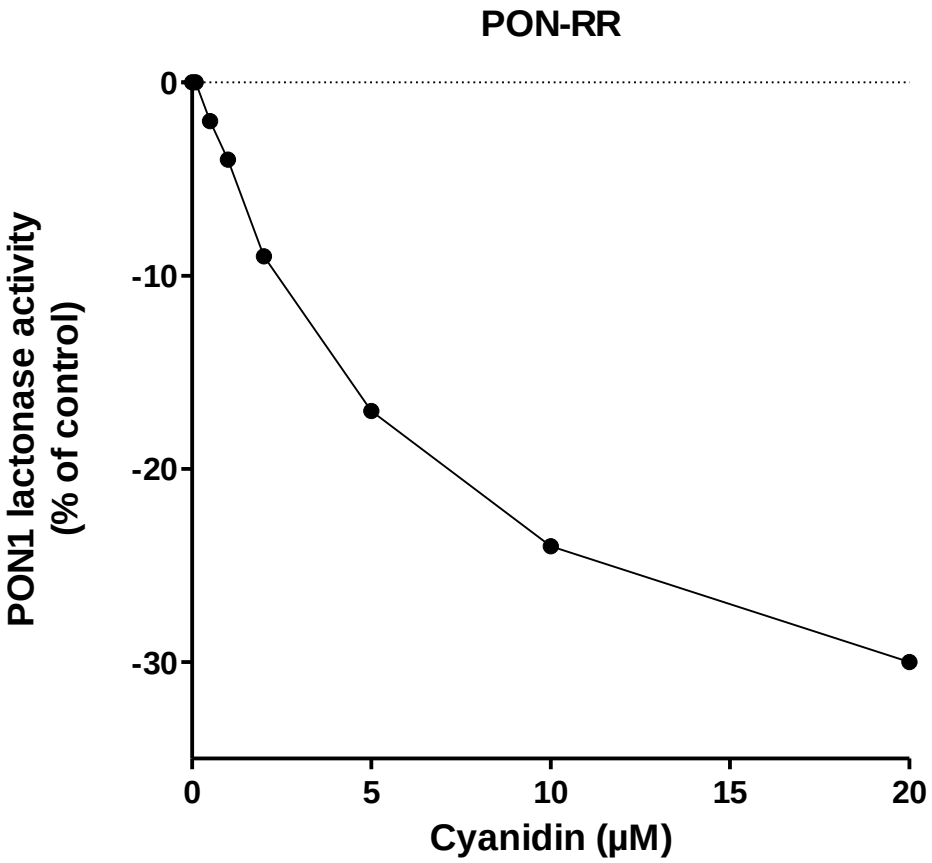


Figure 3. 20: Effect of cyanidin on PON1 (RR) lactonase activity.

PON-RR enzyme was incubated with different concentrations of cyanidin for 10 min at 37°C. TBBL then was added and the activity was measured. The slope of the reaction rate was calculated using the instrument software. The % of change was calculated relative to the control (0.1% DMSO). Treatments were conducted in quadruplicates and the experiments were repeated at least two times.

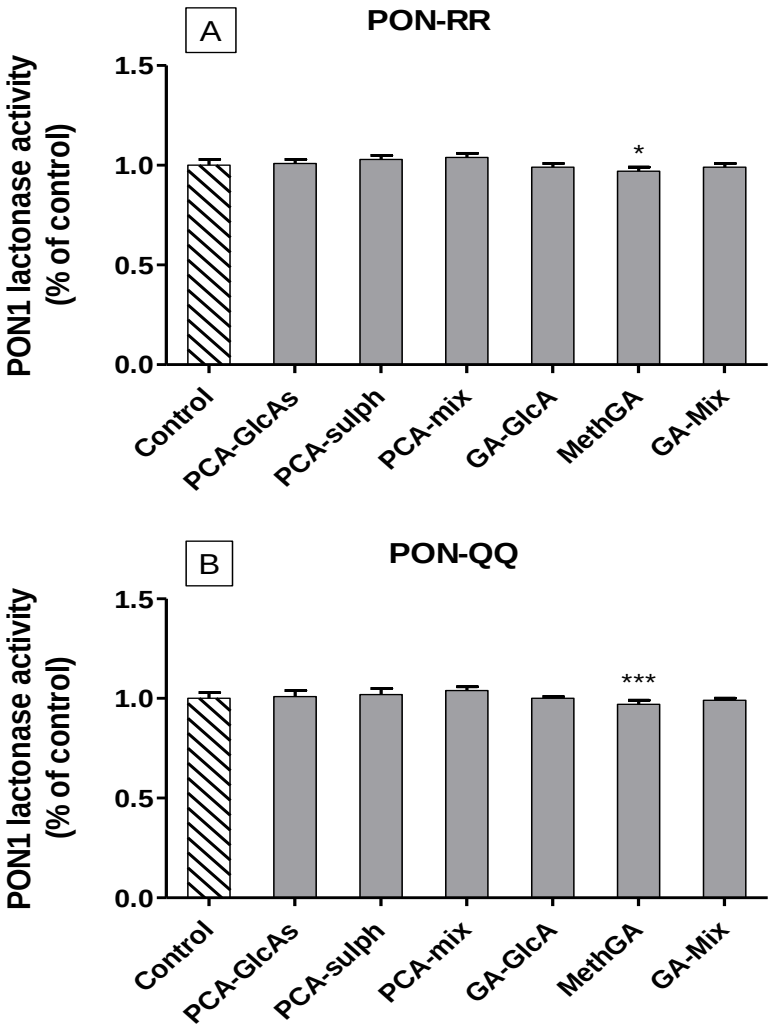


Figure 3. 21: Effect of anthocyanin phase-ii-metabolite conjugates on (A) PON-RR and (B) PON-QQ arylesterase activity.

PON-RR and PON-QQ enzymes were incubated with treatments for 10 min at 37ºC. Control = DMSO, PCA-GlcAs = (1 µM of PCA-3-glucuronide and PCA-4-glucuronide), PCA-Sulphs = (1 µM of PCA-3-sulphate and PCA-4-sulphate), PCA-Mixture = (1µM of C3G, PCA and PCA conjugates), GA-GlcAs = (1 µM of gallic acid-3-glucuronide and gallic acid-4-glucuronide), MethGA = (1 µM of 3-*O*- methylgallic acid and 4-*O*- methylgallic acid), GA-Mixture = (1 µM of D3G, gallic acid and gallic acid conjugates). The slope of the reaction rate was calculated using the instrument software. The % of change to the slope was calculated relative to the control. Data are shown as means ± SD. * $p \leq 0.05$ and *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were conducted in quadruplicates and the experiments were repeated at least two times.

3.6. Discussion

The aim of the research described in this chapter was to investigate whether anthocyanins and their metabolites are capable of increasing PON1 activities which would thereby be consistent with the reported observation of an inverse association with increased risks of cardiovascular diseases. Therefore, the experiments reported in this chapter were designed to investigate a number of hypothese: (1) Anthocyanins and their metabolites cause increases in PON1 gene expression and therefore its activities; (2) anthocyanins and their metabolites would act as activators for PON1 itself and thereby increase its enzyme activities. In addition, it was planned to investigate the effect of anthocyanins and their metabolites on the process of PON1 enzyme secretion using HepG2 as a cell model. But, since PON1 secretion could not be demonstrated in these cells, this could not be investigated.

The data presented in this chapter show that: (1) none of the tested anthocyanins or their tested metabolites increased PON1 promoter activity; (2) none of these compounds significantly affected PON1 arylesterase activity or lactonase activity with either PON1 phenotypes, PON-RR or PON-QQ; except for some small effects that likely lack clinical importance; (3) cyanidin decreased the lactonase activity of PON-RR in a dose-dependent manner.

The novelty of this research is that it is the first study to investigate the effects of anthocyanins and their metabolites individually and in mixtures on activation of the PON1. Gallic acid, ellagic acid, punicalagin, naringenin, resveratrol, quercetin, catechin and isorhamnetin are the only pure phenolic compounds that have been investigated for their effects on PON1 gene expression or enzyme activity and reported so far. [254], [279]–[282], [291]–[293]. This study is the first to consider investigating the effect of anthocyanins and their metabolites directly on PON1 lactonase activity which is the native activity of PON1. To the best of my knowledge, there was not any study investigated the effects of any other polyphenolic compounds on lactonase activity.

It has been previously reported that anthocyanin consumption was able to increase PON1 arylesterase activity in humans and this was associated with improved antioxidant abilities of HDL [170]. This finding and others suggest that the increase of PON1 activity is a potential novel mechanism of anthocyanin to modulate cholesterol metabolism and therefore reduce CVD risk. However, the underlying molecular mechanisms by which anthocyanins induced PON1 activity have yet not been fully understood. Therefore, it was hypothesised here that anthocyanins and

their metabolites would increase PON1 by upregulating of gene expression, activating the enzyme itself, and/or enhancing the enzyme secretion process.

3.6.1. Effect of anthocyanins and their metabolites on PON1 promoter activity.

To investigate the effect of anthocyanins and their metabolites on PON1 gene expression, an PON-Huh7 cell line has been used as an *in vitro* model. PON1-Huh 7 is a liver cell line that is stably transfected with a PON1 promoter. Since PON1 is secreted from liver, using a liver cell line is more relative to answer the question. The reason of not using normal non-transfected liver cell line is that the only available cell line was HepG2 which was tested and it was found that it did not produce any PON1 and the gene expression was very low which make it hard to use it as model for PON1 gene expression or secretion. On the other hand, PON1-Huh7 has been successfully used in several studies with phenolic compounds [275], [279], [281], [282], [291], [292]. Reporter gene assays have been extensively used for the same purpose [278]. As shown in the results, none of the anthocyanins and their metabolites significantly changed PON1 promoter activity at any of tested concentrations. Apart from gallic acid, none of these tested compounds have been reported before. Gallic acid reported to increase PON1 promoter activity by 8.5-fold which is inconsistent with the present study[291]. This contradiction may be attributed to the difference in concentration of gallic acid. In the study that reported a positive effect of gallic acid [291], the concentration was 360 µM which was about 36 times higher than the highest concentration in the present study which may explain why gallic acid could not induce PON1 promoter activity in the current project. In addition, most of the other reported non-anthocyanins phenolic compounds such as catechin, quercetin, punicalagin, ellagic acid at concentrations similar to what was tested in the current study did not affect PON1 gene expression or PON1 promoter activity which support the current findings. [281], [291], [292]. On the other hand, curcumin, the positive control increased PON1 promoter activity which was consistent with previous reports [278], [282], [290]. This finding confirm that the model was working fine, and the non-significant change was real.

Regarding curcumin, it was chosen as a positive control for different reasons. First, because the previously reported effect on PON-Huh7, the model used in this experiment [278], [282], [290]. Secondly, because the chemical structure as curcumin is a phenolic compound and consists of two ferulic acid units, one of the main metabolites of C3G [290]. PON1 induction by curcumin seems to be mediated by an aryl hydrocarbon receptor-dependent signal transduction pathway [282]. Although,

the concentration of curcumin is higher than what detected in humans, curcumin was not cytotoxic to PON1-Huh7 cells at concentrations of up to 20 μmol/l [282].

Since the treatments did not affect the gene expression, it was hypothesized that anthocyanins may act as an activator for PON1. Therefore, the effects of anthocyanins and their metabolites on enzyme activities was investigated.

3.6.2. Effect of anthocyanin and their metabolites on PON1 enzyme activities

Many small molecules can act as activators or inhibitors of PON1 [294]. Anthocyanin metabolites are small molecules and may have the potential to activate PON1. For this purpose, two phenotypes of purified PON1, PON-QQ and PON-RR, have been used as a model to test the effect of anthocyanins and their metabolites on PON1 activity. The current study is the first study to look for the direct effect of anthocyanins and their metabolites on PON1 enzyme activity. In addition, this is the first study that considered the differences between PON1 genotype by using two different PON1 phenotypes that isolated from individuals with Q192Q and R192R genotype. Naringin is the only pure flavonoids that has been tested for its direct effect on PON1 activities without considering the genetic background [254]. As shown in the results, except for the high concentration of cyanidin, none of the tested parent anthocyanins compounds and none of the tested metabolites has strong effect on PON1 activities, although some treatment had a very small significant effect. In addition, lactonase activity, the PON1 native activity, seemed to be more affected by anthocyanins treatments than arylesterase. For instance, C3G treatments significantly increased lactonase activity with PON-QQ and PON-RR, while, the same treatments have no effect on arylesterase. Lactonase activity is more important in terms of anti-atherogenicity and apparently anthocyanins treatments affected it positively, although the effect was minor. The possible explanation of null/low positive effect could be using purified enzymes instead of serum. The purified enzyme lacks the native environment such as the presence of HDL and ApoA1 protein which may affect the enzyme structural conformation and its stability and therefore its activity [294]–[298]. The reason for not using serum as a source of the enzyme is that the serum may contain wide range of compounds such as lipoproteins that may bind with anthocyanins and conceal or inhibit its effect. In addition, the present study aimed to compare between two different PON1 phenotype which is difficult to obtain by using serum sample instead.

On the other hand, cyanidin unexpectedly decreased PON1 lactonase activity in PON-RR but not PON-QQ in a dose-dependent manner. The low concentrations,

however, have no effect on the lactonase activity. The arylesterase remained unchanged after cyanidin treatment which confirm that lactonase was the main target of anthocyanins. In fact, cyanidin was not detected in serum in humans after consumption of ^{13}C-labelled C3G. Only traces of cyanidin conjugates were detected in urine in the same study suggesting that the existence of cyanidin in serum in concentrations higher than 0.1 µM is not achievable which likely making the effect of cyanidin on PON1 physiologically unimportant [73]. Furthermore, cyanidin is extremely unstable at high pH with about 50% instantaneous loss of the parent structure at pH 7.4 which was lower than the pH of the assay buffer (pH=8) suggesting that the reduction in PON1 activity was not mediated directly by cyanidin but may be through one or more of its intermediates [41]. In contrast, C3G, the glycoside form of cyanidin, slightly increased PON1 lactonase activity in both PON-RR and PON-QQ. Fortunately, C3G is found in serum in concentrations higher than cyanidin after anthocyanins consumption making the negative observed effect of cyanidin on lactonase insignificant.

Although the effect of anthocyanins on lactonase activity, the physiological activity of PON1, was investigated in this chapter, the main role of anthocyanins in helping PON1 in prevention of LDL oxidation remained undiscovered. Anthocyanins may improve the ability of PON1 indirectly to prevent the oxidation of LDL. Studying the oxidation status of LDL before and after treatment with enzyme combined with anthocyanins may answer this question. Therefore, further investigation is still needed in this aspect. Additionally, the effect of anthocyanins and their metabolites on PON1 secretion and protein level still yet to be investigated.

Therefore, based on the results presented in this chapter, the tested anthocyanins and their tested metabolites have no strong significant effect on PON1 activities and gene expression. This result indicating that anthocyanins and their metabolites did not affect RCT pathway biomarker. However, anthocyanins may target different pathways related to atherosclerosis. To investigate this further, non-targeted approach such as RNA-sequencing can be used to detect which genes and pathways related to atherosclerosis and lipids metabolism were affected by anthocyanin treatments. Therefore, a transcriptomic analysis has been done and is presented in next chapter.

3.7. Conclusion

The results presented in this chapter demonstrate that none of the anthocyanin compounds or their metabolites significantly changed PON1 promoter activity nor strongly changed the arylesterase or lactonase activities regardless PON1 phenotypes. However, the effect of these compounds on PON1 protein level or secretion and on prevention of LDL from oxidation is not excluded. Future work still needed to investigate more untested metabolites, study the effects on LDL oxidation stats and on PON1 secretion. In addition, different atherosclerotic-related pathways need to be investigated. The next chapter therefore will use RNA transcriptomic analysis to discover pathways targeted by anthocyanins.

CHAPTER FOUR

Chapter four: The atherosclerosis-linked pathways and the possible direct molecular targets affected by the anthocyanin metabolites

4.1. Abstract

Data presented in chapter two and three in this thesis showed no significant effects of anthocyanins and their metabolites on key genes of RCT and biomarkers of HDL function in relation to atherosclerosis. However, atherosclerosis is a multifactorial disease that involves various genetic and environmental factors. To enhance our understanding of underlying molecular mechanisms and to discover new biomarkers and pathways of atherosclerosis that might be affected by anthocyanins and their metabolites, an RNA sequencing (RNA-seq) study was conducted. Preloaded-THP-1 macrophages were treated with 10 μM of phloroglucinaldehyde (PGA), syringic acid, vanillic acid and a mixture of them (3.3 μM each) in addition to DMSO as a vehicle control. RNA sequencing was performed using NovaSeq and 100 bp paired end reads. The differential expression analysis of sequencing data revealed no significant changes in gene expression to an adjusted *p*-value ≤0.05. However, PGA significantly changed the expression of 377 genes at adjusted *p*-value ≤ 0.3. Among those, 231 genes were downregulated (average fold change 0.7-fold), and 146 genes were upregulated (average fold change 1.3-fold). Most of these were categorized as non-atherosclerotic-linked genes. The functional enrichment analysis also showed that anthocyanin metabolites regulated pathways involved in DNA repair, cell cycles, inflammatory process, adipogenesis and cancerous-relevant pathways. In conclusion, the result from RNA seq showed no substantial effects of anthocyanin metabolites on the differentially expressed genes or pathways related to atherosclerosis. The lack of effect could be that the most beneficial metabolites have not been examined as only three metabolites were tested in this study or may due to using cell line that is not a target of anthocyanins and therefore future work is required to test more metabolites with several cell lines involved in atherosclerosis.

4.2. Introduction

Atherosclerosis is a commonly described as multifactorial disease involves various genetic and environmental factors as well as conventional risk factors such as dyslipidemia, diabetes and hypertension [299], [300]. Several epidemiological studies revealed that the consumption of anthocyanins is strongly correlated with reduced risk factors of atherosclerosis [116], [119]. Additionally, human and animal intervention studies conducted with anthocyanin-rich fruit and extract showed a protective effects of anthocyanin against several risk factors of atherosclerosis [15], [164]. Although some of these studies provided evidences about the antiatherogenicity properties of anthocyanin such as improvement in lipid profile, modulation of vascular function and increase in the anti-inflammatory properties [15], [301], [302], the underlying mechanisms are not yet fully understood.

There are several *in vitro* experiments were undertaken to explore the molecular mechanisms implicated in bioactivity of anthocyanins using targeted approaches which allow studying specific molecular mechanisms. In some of these studies, pure anthocyanin and its metabolites as well as anthocyanin-rich extracts showed that anthocyanins altered many genes and biomarkers related to atherosclerosis such as genes encoding the cholesterol transporters, scavenger receptors, cell adhesion molecules, vasoconstrictor ET-1 [45], [250], [303], [304]. However, the molecular targets of anthocyanins and their metabolites still largely unknown due to using pure compounds with concentrations higher than the physiologically relevant concentrations or using whole material extracts that contain other bioactives. In chapter two and three in this thesis, the effect of wide range of pure anthocyanin and their metabolites at physiologically relevant concentration on several biomarkers related to RCT and HDL function was investigated with no significant changes in any of these biomarkers. However, anthocyanin may target different genes and pathways suggesting using non-targeted approaches to identify them. The global genomic approaches such as microarray technology or next generation sequencing (NGS) technology allow studying the effects on the global gene expression which provide a better insight into the disease mechanisms and the possible interaction between different pathways especially in complex multifactorial diseases like atherosclerosis [249], [305], [306].

The beneficial effects of anthocyanins were also supported by several reports that studied the global gene expression using microarray. In a very recent study, the impact of consuming 11 g wild blueberry powder (containing 150 mg of anthocyanins)

for 28 days on the expression of miRNAs in human was analysed using Human miRNA Microarray [307]. The data has shown that 608 genes were significantly differentially expressed. In addition, there was 35 significant gene networks identified following the functional annotation using gene ontology. Among them, 11 are known to be involved in the regulation of chemotaxis and inflammation/immune response, 9 in cell adhesion and cytoskeleton organization, and few networks regulating signal transduction, apoptosis, or development [307].

In mice, there are several studies investigated the effect of anthocyanin on differential expression. Mauray *et al.* (2010) reported that the consumption of bilberry extract for two weeks altered the expression of 2,289 genes involved in various molecular pathways such as cholesterol metabolism, inflammatory processes and cell adhesion in liver tissue of apo $E^{-/-}$ mice [308]. In another study, bilberry extract changed 1261 genes in aorta tissue of apo $E^{-/-}$ mice. The modulated genes were involved in inflammatory response, cell proliferation through vascular endothelial growth factor (VEGF), cell adhesion and lipid metabolism [45]. Moreover, the effects of bilberry extract (75 μg/ml) on genes expression profile were also investigated in mouse macrophages. In this study, the bilberry extract downregulated about 2000 genes by 2-fold. Among of them, protein coding genes for liposaccharide synthesis (LPS), tumour necrosis factor (TNF), prostaglandin-endoperoxide synthase (PTGS), Tenascin (TNC) , Interleukin -1β and -6 (IL-1β and IL-6), and cyclooxygenase2 (COX-2) which attenuate the atherosclerosis [309]. Furthermore, in a study where adipocytes were isolated from human and rat that were treated with 100 μM of C3G and cyanidin, a reduction in the expression of pathways related to inflammatory process, cell adhesion and lipid metabolism were reported using microarray genes profiling [310], [311].

Although the global gene expression analysis in these studies supported the notion that anthocyanin demonstrates protective effects against risk factors of CVD and provided insight into some possible targeted pathways, the underlying mechanisms are still inconclusive as whole materials that contain other bioactives were used. Additionally, these studies did not investigate the effect of anthocyanin metabolites or examined the effects at high concentrations. Therefore, more transcriptomic studies are needed to investigate this further.

High-throughput sequencing technologies such as RNA sequencing (RNA seq) have become more popular alternative to microarrays technologies [312]. Unlike microarray, RNA seq does not require specific probes allowing detection of novel transcripts, alternative splicing junctions, new single nucleotide variants, allele-

specific expression and other changes that arrays cannot identify [313]–[315]. Compared to microarrays, the high specificity and sensitivity of RNA seq allow detecting the expressed genes in the whole genome and detecting low expressed genes in more accuracy than microarray that require hybridization [312]. With all the previous advantageous and many others, RNA seq provide a powerful tool for discovering and profiling the transcriptome with deep information that allow exploring and predicting biomarkers and help understanding the underlying molecular mechanisms of interaction between diet and diseases, especially the complicated one such as atherosclerosis. Therefore, RNA seq transcriptomic study was conducted as explore the molecular mechanisms and the possible targeted by which anthocyanin deliver its action.

4.3. Objectives

The main aim of the research presented in this chapter was to perform RNA seq transcriptomic study to investigate the influence of C3G and D3G main metabolites on (1) the transcriptome profiling, (2) the differentially expressed genes and (3) the functional pathways using human macrophages cell line to gain better insight into the molecular mechanisms by which anthocyanins and their metabolites deliver their effects.

4.4. Materials and methods

4.4.1. Cell culture

THP-1 human monocyte cells were routinely maintained and cultured in bicarbonate RPMI-1640 media as described previously in section 2.4.1. THP-1 monocyte cells were seeded at 5 x 10^5 cell/ml in 12-well plates and differentiated by 100 ng/ml PMA for 48h followed by preloading step with ac-LDL (25 µg/ml) for additional 24 h and prior to the treatments.

4.4.2. Treatments

Preloaded THP-1 macrophages were treated with control (DMSO 0.1%), 10 µM of PGA, vanillic acid, syringic acid individually and a mixture of them with 3.3 µM each for 24 hr. The stock solutions as well as the treatments were prepared as described in section 2.4.2. After treatment, the cells were subjected to RNA extraction.

4.4.3. RNA extraction

RNA extracted from treated cells as previously mentioned in section 2.4.3. Briefly, the cell layer was washed twice with cold PBS and subjected to RLT extraction buffer. RNA extract then was collected and homogenised using QIAshredder prior to purification with RNeasy® Mini kit. RNA quantity and purity were measured using the nanodrop. Three readings for RNA quantification were obtained per sample and the average of RNA concentration was then calculated. RNA samples were aliquoted and stored at -80°C until using.

4.4.4. RNA integrity assessment

An aliquot of RNA sample was used to assess the quality of RNA using an Agilent RNA 6000 Nano LapChip kit (Cat # 5067-1511) and an Agilent 2100 Bioanalyzer according to the manufacturing protocol. RNA integrity using this method is simply measured using chip system that allow quick electrophoresis separation. There are microchannels within the chip that should be filled with a mixture of fluorescence dye and a sieving polymer that allow a separation of RNA fragments based on their size. The fluorescence dye binds with RNA fragment producing fluorescent signal that can be measured and plotted graphically. To measure the size and the concentration of RNA fragments, RNA 6000 ladder standard was run alongside RNA samples on every chip. The software automatically compares the size and the concentration of the unknown RNA samples with the reference ladder and to identify the ribosomal RNA (rRNA). RNA integrity (quality) was then calculated for each sample based on the ratio of the 28S and 18S rRNA fragments. High RNA quality samples can be characterized by two distinguished peaks corresponding to 28S and 18S rRNA and flat baseline especially between 28S and 18S bands. 28S peak should be larger than 18S. The software automatically assesses RNA quality based on the electrophoretic RNA measurement using a number called RNA Integrity Number (RIN) which range from 1 to 10 where 1 is completely degraded RNA, while 10 being the most intact RNA. Fig 4.11 shows an example of high integrity RNA sample.

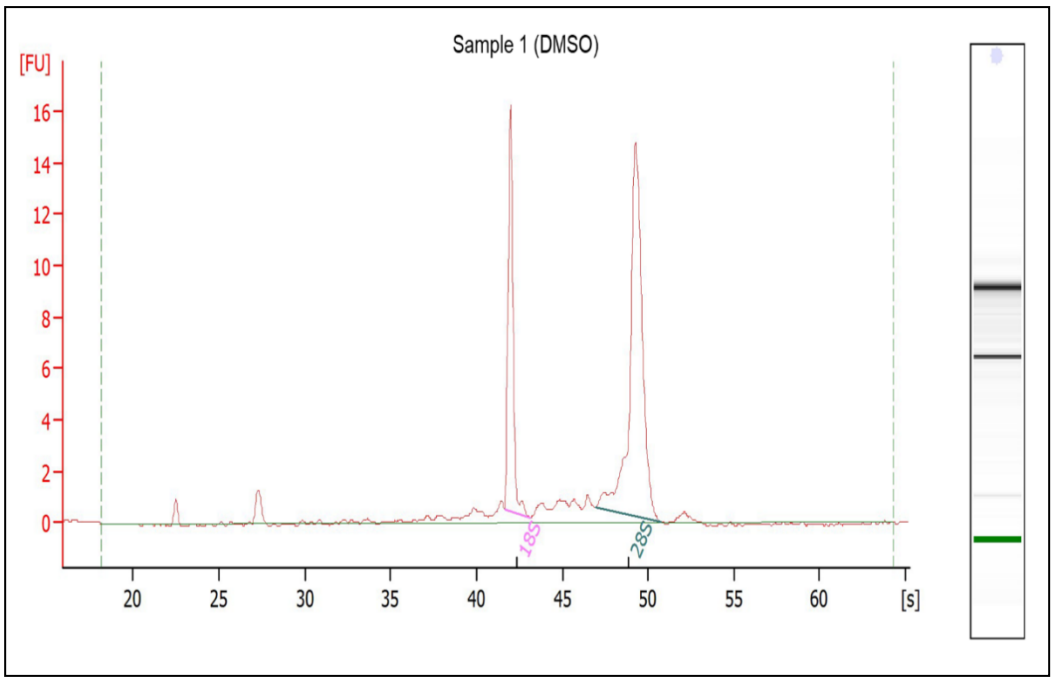


Figure 4. 1: An electropherogram of high-quality RNA sample analysed by Agilent 2100 Bioanalyzer.

RIN = 9.4, 18S rRNA area is 23.3 % of total area, 28S rRNA is 46.5 % of total area.

4.4.5. RNA sequencing

After RNA quality assessment, 20 samples (5 treatments x 4 replicates) with RIN higher than 8 were sent for sequencing by Macrogen Human Genomics Ltd (https://dna.macrogen.com/eng/index.jsp) in Rep of Korea using Illumina sequencing. The company was responsible for quality control, libraries preparation and sequencing.

The libraries were first constructed using TruSeq RNA (Poly-A) library preparation kit according to the manufacturing protocols. This kit is stranded mRNA sample preparation kit that generate mRNA-focused sequencing libraries from total RNA. The basic of this kit is simply purifying the poly-A containing mRNA molecules using poly-T oligo attached magnetic beads. The mRNA was then fragmented and copied into cDNA library using reverse transcriptase and random primers which then ligated into adaptors to create the final cDNA library. The libraries were then sequenced using

the Illumina NovaSeq and 100 bp paired end reads were generated with average of 40 million reads per samples.

4.4.6. Data processing

The sequences raw data was provided in FASTQ file format. The data processing and analysis was performed by a bioinformatician in house according to the protocol described elsewhere [316]. Briefly, the analysis of RNA seq data started with the pre-processing of the raw sequencing reads to remove ribosomal reads using SortMeRNA v 2.1b [317]. The next step was to remove adaptor sequences, which was done with a stringency of a minimum of 5 bases overlap. Then, for quality control, low quality reads (< 30) were removed as well as short reads (< 60 bp). To remove adaptor sequences and for quality control, TrimGalore v 0.4.2 software was used(https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The high-quality filtered reads were then aligned to the human reference genome (ensemble release 84, GRCh38, ftp://ftp.ensembl.org/pub/release-84/fasta/homo_sapiens/dna/Home_sapiens.GRCh38.dna.primary_assembly.fa) using the splice-aware aligner HISAT2 v 2.0.5 [318]. These alignments were then assembled into full-length transcripts and quantified in each sample using String Tie v 1.3.3 [319]. String Tie estimates the expression levels of all genes and transcripts. Finally, the read alignments and merged transcripts were used to re-estimate abundances when necessary to create transcript and gene counts for further analysis differential expression.

4.4.7. Differential expression analysis

The raw counts of genes were transformed to counts per million (CPM) and log CPM which then filtered to remove the low expressed genes prior to normalization and unsupervised clustering. The transformation, normalization and differential expression analysis was performed using Limma (v 3.38.3), edgeR (v 3.24.3) in RStudio [320]–[322]. The ranked genes subjected to gene set enrichment analysis (GSEA) using The Molecular Signatures Database (MsigDB) hallmark database to identify the enriched pathways [323].

The differential expression analysis was performed using adjusted *p*-value and Benjamini-Hochberg multiple testing correction. adjusted p-values were used to identify differentially expressed genes. A *p*-value ≤ 0.05 was considered significant. Four biological replicates of each treatment were conducted with total 20 samples were sequences and analysed

4.5. Results

4.5.1. Quality control for RNA sequence data

In order to understand the possible target of anthocyanin metabolites, a transcriptomic analysis of THP-1 was carried out. Preloaded THP-1 macrophages were treated with10 μM of PGA, syringic, vanillic acid; or a mixture of these three (3.3 μM each) and the result was compared to vehicle control (DMSO). First, high-quality RNA samples should be used for sequencing and therefore, RNA integrity was assessed using the bioanalyzer. The quality of all twenty RNA samples was very high and the RIN number varied from 9 to 10 which was enough to access library preparation and sequencing process using Illumina NovaSeq to generate paired-end (100 bp) platform. In total, an average of 48,172,840 ± 7,230,344 non-paired-end reads were obtained. The average total paired-end reads were 24,086,420 ± 3,615,172 (Table 4.1) The smallest library was 20,009,537 (40,019,074 non-paired-end) reads and the largest library was 29,607,296 (59,214,592 non-paired-end) reads. First, rRNA reads were removed. Since the libraries was constructed using TruSeq RNA (Poly-A) technology, the non-rRNA reads (mRNA) represented 99.1 ± 0.2 % of total reads (average of 23,963,981 ± 3,597,631 paired-end reads). After adapters sequences trimming and discarding low quality reads (reads shorter than 60 bp), an average of 23,920,067 ± 3,593,971 high quality clean paired-end reads were obtained which represent about 99.8 % of total non-rRNA reads (Table 4.1).

High quality reads where then mapped to the reference genome using HISAT 2 software. A large number of clean reads which represent an average of 97.3 ± 0.3 of total reads were successfully aligned (Table 4.1) which was consistent with what was reported previously with high-quality datasets [324], [325].

4.5.2. Differentially expressed genes

After calculating the gene counts using StringTie software, the output file is a matrix with raw counts. In gene expression raw counts cannot be used for the analysis. Instead the raw counts are transformed onto a scale that accounts for the library size differences [322], [326]. For RNA-seq data in this study, the data was transformed into counts per millions (CPM) and log-CPM which then used for differential expression analysis.

Table 4. 1: Summary of quality characteristics of RNA sequencing reads before and after processing

Treatment	Raw data	Filtering rRNA and quality control			Mapping		
	Total read (Paired-end) [1]	Non-rRNA reads (Paired-end)	Reads after adapter trimming and QC [2]	Clean reads (%)[2]	Uniquely aligned reads	Multi-mapped reads	Overall alignment
control	28,164,254	28,048,609	27,991,331	99.8	22,640,305	3,823,649	97.0
control	29,607,296	29,496,260	29,443,480	99.8	23,853,415	4,033,492	97.1
control	20,463,816	20,360,355	20,301,988	99.7	16,462,979	2,735,237	97.3
control	23,446,159	23,334,686	23,290,828	99.8	18,937,973	3,200,771	97.5
Vanillic acid	28,554,853	28,368,366	28,311,277	99.8	22,838,966	3,843,615	96.8
Vanillic acid	20,465,809	20,332,956	20,291,633	99.8	16,558,090	2,711,796	97.3
Vanillic acid	20,152,652	20,014,782	19,957,143	99.7	16,244,479	2,697,930	97.2
Vanillic acid	29,400,022	29,246,167	29,198,440	99.8	23,679,905	3,984,232	96.9
Syringic acid	22,941,377	22,806,237	22,747,048	99.7	18,492,026	3,098,335	97.3
Syringic acid	27,907,865	27,715,962	27,669,083	99.8	22,495,511	3,715,977	97.0
Syringic acid	21,418,009	21,238,580	21,170,387	99.7	17,185,728	2,909,678	97.3
Syringic acid	22,493,356	22,373,270	22,334,872	99.8	18,231,297	3,062,658	97.7

- ([1]) Paired-end = sequence both ends of a DNA fragment, total read = paired-end reads x 2 both reverse and forward sequence;
- ([2]) QC = quality control, % of clean read is reads after adapter trimming and QC divided by non-rRNA paired-end reads.

Table 4.1: continued

Treatment	Raw data	Filtering rRNA and quality control			Mapping		
	Total read (Paired-end) [1]	Non-rRNA reads (Paired-end)	Reads after adapter trimming and QC [2]	Clean reads (%)[2]	Uniquely aligned reads	Multi-mapped reads	Overall alignment
PGA[3]	26,736,887	26,539,520	26,493,195	99.8	21,450,870	3,606,728	96.9
PGA	20,015,254	19,941,386	19,916,164	99.9	16,267,642	2,679,859	97.5
PGA	28,034,259	27,939,984	27,906,229	99.9	2,275,746	3,874,849	97.0
PGA	21,539,828	21,448,060	21,420,582	99.9	17,453,835	2,956,037	97.6
mixture	20,009,537	19,948,650	19,927,256	99.9	16,334,782	2,694,368	97.7
mixture	27,006,383	26,903,599	26,870,744	99.9	21,950,897	3,648,409	97.4
mixture	20,466,052	20,386,827	20,362,707	99.9	16,712,065	2,724,257	97.7
mixture	22,904,740	22,835,371	22,809,038	99.9	18,700,808	3,106,361	97.8

- ([1]) paired-end = sequence both ends of a DNA fragment, total read = paired-end reads x 2 both reverse and forward sequence;
- ([2]) QC = quality control, % of clean read is reads after adapter trimming and QC divided by non-rRNA paired-end reads.
- ([3]) PGA = phloroglucinaldehyde, control = DMSO.

Based on CPM, 56662 features (genes) were identified which include a mix of expressed and lowly expressed genes. The genes that are lowly expressed or unexpressed throughout all samples interfere with some of the statistical approximations that are used in the pipeline and therefore, it is recommended to filter them [326]. Using edgeR package and CPM threshold 0.4 which means that the gene is considered expressed if at least 12 counts in the smallest libraries and 18 counts in the largest libraries in at least 4 samples were identified. Based on that, the number of expressed genes after filtering were 17,233 genes. Fig 4.2, demonstrates the difference between pre-filtered and post-filtered.

These genes were normalized using trimmed mean of M-value to identify the differentially expressed genes. Using cutoffs of adjusted *p*-value less than 0.05, only one gene and two genes were differentially expressed after syringic acid and PGA treatment, respectively. Therefore, the analysis was repeated using higher adjusted *p*-value (≤ 0.3).

As shown in Fig 4.3, PGA was the only treatment that showed an effect on the differential gene expression with total of 377 genes were differentially expressed; 231 genes were downregulated, and 146 genes were upregulated at adjusted *p*-value ≤ 0.3. Syringic acid, on the other hand, changed the expression of six genes compared to the control; four genes were downregulated, and two genes were upregulated at the same adjusted *p*-value. Whereas, there was no significant differentially expressed gene after vanillic acid or mixture in comparison with control treatment. The genes that were differentially expressed after PGA treatment were ranked according to their fold change. The fold change ranged from 1.07 to 4.79-fold compared to control for upregulated genes, while, the fold change ranged from 0.03 to 0.95-fold for downregulated genes compared to control group. Table 4.2 and 4.3 show the top twenty and the lowest twenty expressed genes.

Most of changed genes were not relevant to lipid metabolism or CVD risk factors. CD36 and Plin2 genes were the only genes that were identified as CVD relevant genes. CD36 is a gene encode for CD36 scavenger receptor protein synthesis, while, Plin2 is a gene encode for lipid droplet protein which is involve in lipid droplet formation. The fold change for both genes were 1.2 and 1.13 for CD36 and Plin2, respectively.

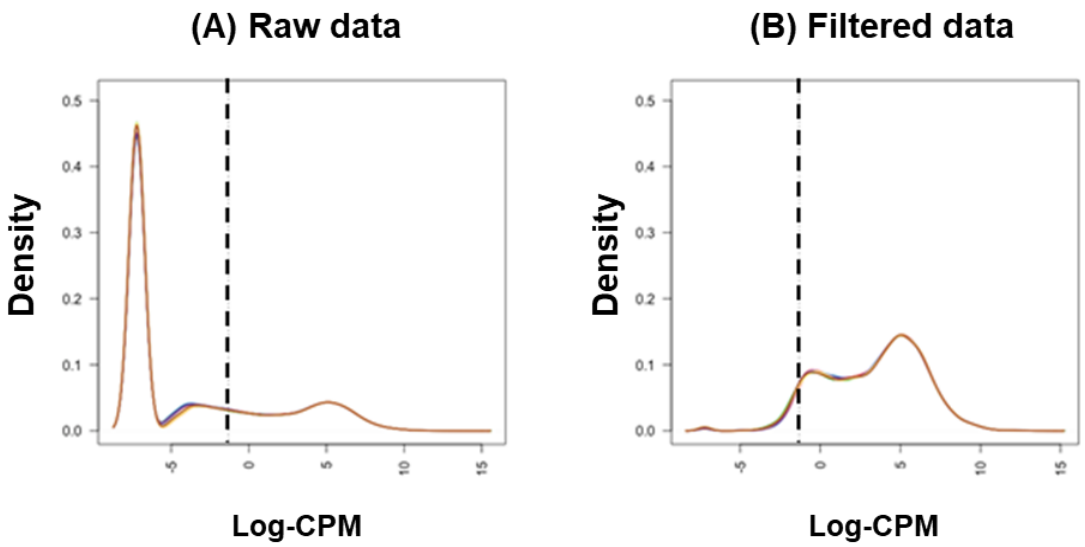
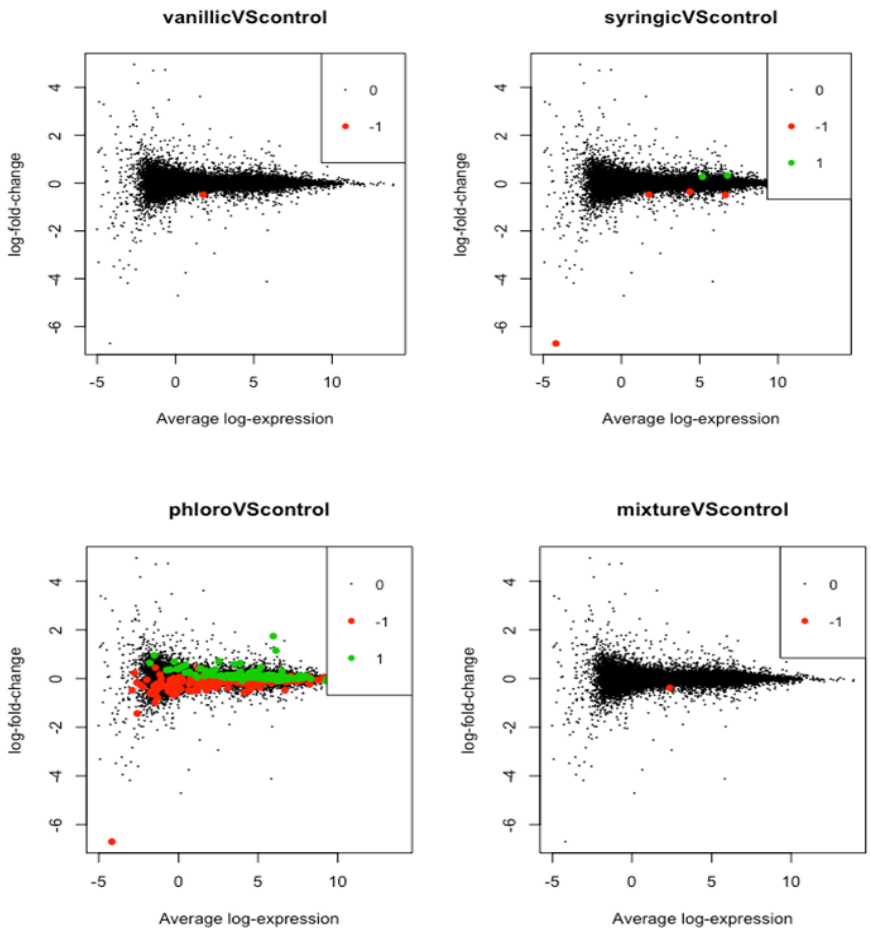


Figure 4. 2: The distribution of Log-CPM for (A) raw data and (B) of filtered data.

Raw data represent all features identified, while filtered data represents the filtered data after removing genes with CPM ≥ 0.4. The dotted vertical lines mark the Log-CPM threshold (Log 0.4 = -0.4). The features that had log-CPM ≤ -0.4 were removed.



	q<0.05		q<0.1		q<0.2		q<0.3	
	Down	Up	Down	Up	Down	Up	Down	Up
Vanillic vs control	0	0	0	0	0	0	1	0
Syringic vs control	1	0	2	2	3	2	4	2
PGA vs control	2	0	4	0	21	11	231	146
Mixture vs control	0	0	0	0	1	0	1	0

Figure 4. 3: Interactive mean-difference plot of expression and a table of number of differentially expressed genes at different adjusted p-value.

Average log-expression = average log-CPM, q = adjusted *p*-value (≤ 0.3), red spots = downregulated genes; green spots = upregulated genes; black spots= genes with no significant difference at q ≤ 0.3. PGA = phloroglucinaldehyde, Control = DMSO. mixture = mixture of syringic acid, vanillic acid, PGA (3.3 µM or each).

Table 4. 2: The top twenty differentially upregulated genes based on fold-change in PGA treatment.

Gene name	Log FC[1]	Adjusted *p*-value	FC [1]	Gene encode [4]
LRRC4	2.26	0.25	4.79	Excitatory synapses
NA[2]	1.30	0.30	2.47	-
ASXL2	1.25	0.22	2.37	ASXL transcriptional regulator 2
LINC00942	1.21	0.30	2.31	Intergenic Non-Protein Coding RNA 942
MAGEA4	1.02	0.30	2.03	Melanoma-associated antigen 4
ZNF35	0.99	0.30	1.98	Zinc finger protein 35
NA	0.93	0.30	1.91	-
AC009303.4	0.90	0.26	1.87	Nuclear factor (erythroid-derived 2)-like 2
AC116347.1	0.87	0.30	1.83	Proteasome activator subunit 2
HHLA3	0.76	0.25	1.70	HERV-H LTR-associating protein 3
CDKL1	0.75	0.30	1.69	Cyclin dependent kinase Like 1
NACA2	0.72	0.26	1.65	Nascent polypeptide associated complex subunit alpha 2
NA	0.64	0.22	1.56	-
DMC1	0.63	0.30	1.55	meiotic recombination protein DMC1/LIM15 homolog
WDR31	0.57	0.30	1.49	WD repeat Domain 31
NA	0.54	0.14	1.45	-
CCNG2	0.52	0.25	1.44	NF[3]
GPT2	0.52	0.26	1.44	NF
AL590764.1	0.52	0.30	1.43	NF
NA	0.52	0.26	1.43	-

([1]) FC = fold change, ([2]) NA = the gene name was not identified, ([3]) NF = the gene function was not found, ([4]) the function of genes were identified using genecards database (https://www.genecards.org/)

Table 4. 3: The twenty most downregulated genes based on fold-change in PGA treatment.

Gene name	Log FC[1]	Adjusted *P-v*alue	FC	Gene encode[4]
NA	-6.97	0.14	0.01	-
C7	-5.18	0.16	0.03	Complement C7
NA[2]	-3.88	0.24	0.07	-
PHLDB2	-3.84	0.23	0.07	Pleckstrin homology like domain family b member 2
UMOD	-3.76	0.25	0.07	Tamm-horsfall urinary glycoprotein
AQP2	-3.46	0.30	0.09	Aquaporin 2
YTHDF2P1	-3.13	0.25	0.11	Yth domain family member 2 pseudogene 1
ALDOB	-3.10	0.26	0.12	Aldolase, fructose-bisphosphate b
ABHD17AP4	-3.09	0.26	0.12	Ab-hydrolase domain containing 17a pseudogene p4
LIMCH1	-2.94	0.26	0.13	Lim and calponin homology domains 1
7SK	-2.88	0.15	0.14	RNA component of 7sk nuclear ribonucleoprotein
AC048382.5	-2.74	0.29	0.15	Novel transcript, antisense to ZSCAN2
RN7SKP203	-2.71	0.20	0.15	Rn7sk pseudogene 203
PCK1	-2.53	0.30	0.17	Phosphoenolpyruvate carboxykinase 1
PTPRM	-2.43	0.28	0.19	Protein tyrosine phosphatase receptor type m
NA	-2.33	0.28	0.20	-
AC021016.3	-2.24	0.30	0.21	Novel transcript, antisense to CTDSP1
RN7SL4P	-2.16	0.13	0.22	RNA, 7sl, cytoplasmic 4, pseudogene
RN7SL5P	-2.11	0.05	0.23	RNA, 7sl, cytoplasmic 5, pseudogene
AC007952.4	-2.06	0.28	0.24	Novel transcript

([1]) FC = fold change, ([2]) NA = the gene name was not identified, ([3]) NF = the gene function was not found, ([4]) the function of genes were identified using genecards database (https://www.genecards.org/)

4.5.3. Functional enrichment analysis.

In order to interpret and identify the relevant biological pathways of the sequenced genes, gene set enrichment analysis (GSEA) was performed using The Molecular Signatures Database (MsigDB) hallmark gene sets.

The hallmark analysis revealed that PGA significantly affected 13 pathways in comparison to control group at adjusted p-value ≤ 0.05. All of them were upregulated (Fig 4.4-A). Apart from adipogenesis pathways [327], none of the changed pathways were atherosclerotic-relevant. For instance, MYC Targets V1 which got the highest normalized enrichment is cancerous-relevant pathway [328], while, G2M Checkpoint pathway prevents cells from entering mitosis when DNA is damaged [329].

When the adjusted p-value increased to ≤ 0.08, two additional upregulated pathways and two downregulated pathways were identified (Fig 4.4-B). One of the additional upregulated pathways is fatty acid metabolism that is particularly relevant to CVD [330], while, epithelial-mesenchymal transition (EMT) was one of the downregulated pathways which is involved indirectly in atherosclerosis. EMT pathway is mainly involved in embryonic development, wound healing, regeneration, heart development and cancer. However, EMT can be described as specialized form of endothelial-mesenchymal transition which involves in pathophysiology of atherosclerosis [331], [332].

On the other hand, vanillic acid barely affected the pathways with Interferon Alpha Response being the only significantly changed pathway in comparison to control group at adjusted p-value ≤ 0.05 (Fig 4.5). Interferon Alpha Response pathway is involved in antiviral response of the host and it is not atherosclerotic-related pathway [333].

In contrast, syringic acid upregulated 13 gene sets with adjusted *p*-value ≤ 0.05 (Fig 4.6). Although, none of the upregulated pathways are involved in lipid/lipoprotein metabolism or RCT, two of the upregulated pathways are involved in inflammation process; tumour necrosis factor alpha (TNFα) via NF/HB and inflammatory response pathways (Fig 4.6-A). Additionally, syringic acid upregulated EMT pathway. These three pathways may be involved indirectly in atherosclerosis.

Furthermore, a mixture of PGA, vanillic acid, syringic acid (3.3 μM of each) upregulated only two pathways, MYC Targets V1 and G2M Checkpoint pathways at adjusted *p*-value ≤ 0.05 (Fig 4.7-A). These two pathways were also upregulated by PGA and they are not atherosclerotic-related pathways. On the other hand, using adjusted *p*-value ≤ 0.1 revealed an additional 9 pathways that were upregulated by

the mixture treatment (Fig 4.7 A&B). However, none of them were atherosclerotic-linked pathways. Moreover, none of the dysregulated pathways were downregulated.

The enrichment analysis was also conducted using Reactom and Gene Ontology databases (Appendix 1) without a big difference in changed pathways with Hallmark database.

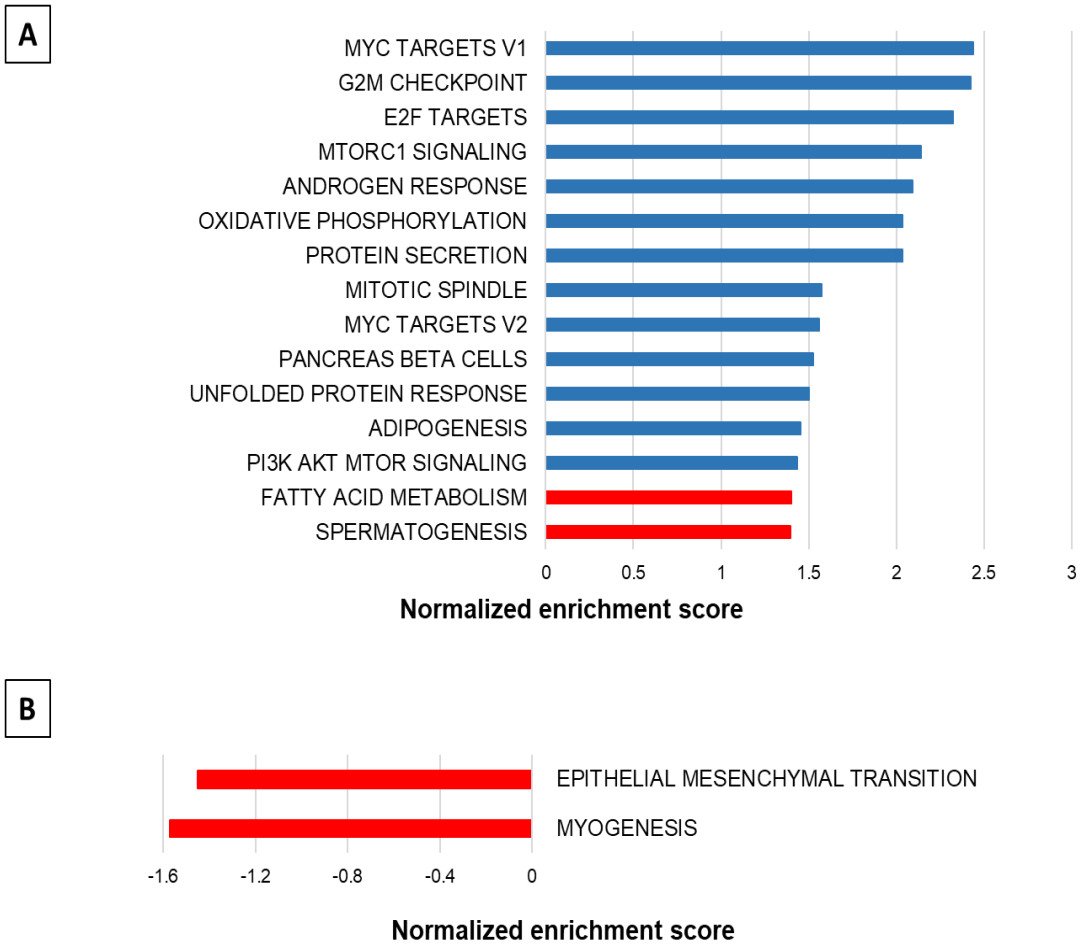


Figure 4. 4: Hallmark pathway analysis of genes expressed by PGA treatment, (A) upregulated and (B) downregulated pathways.

Blue bars represent the significantly changed pathways by PGA in comparison with control group at adjusted *p*-value ≤ 0.05; red bars represent the significantly changed pathways at adjusted *p*-value ≤ 0.08.

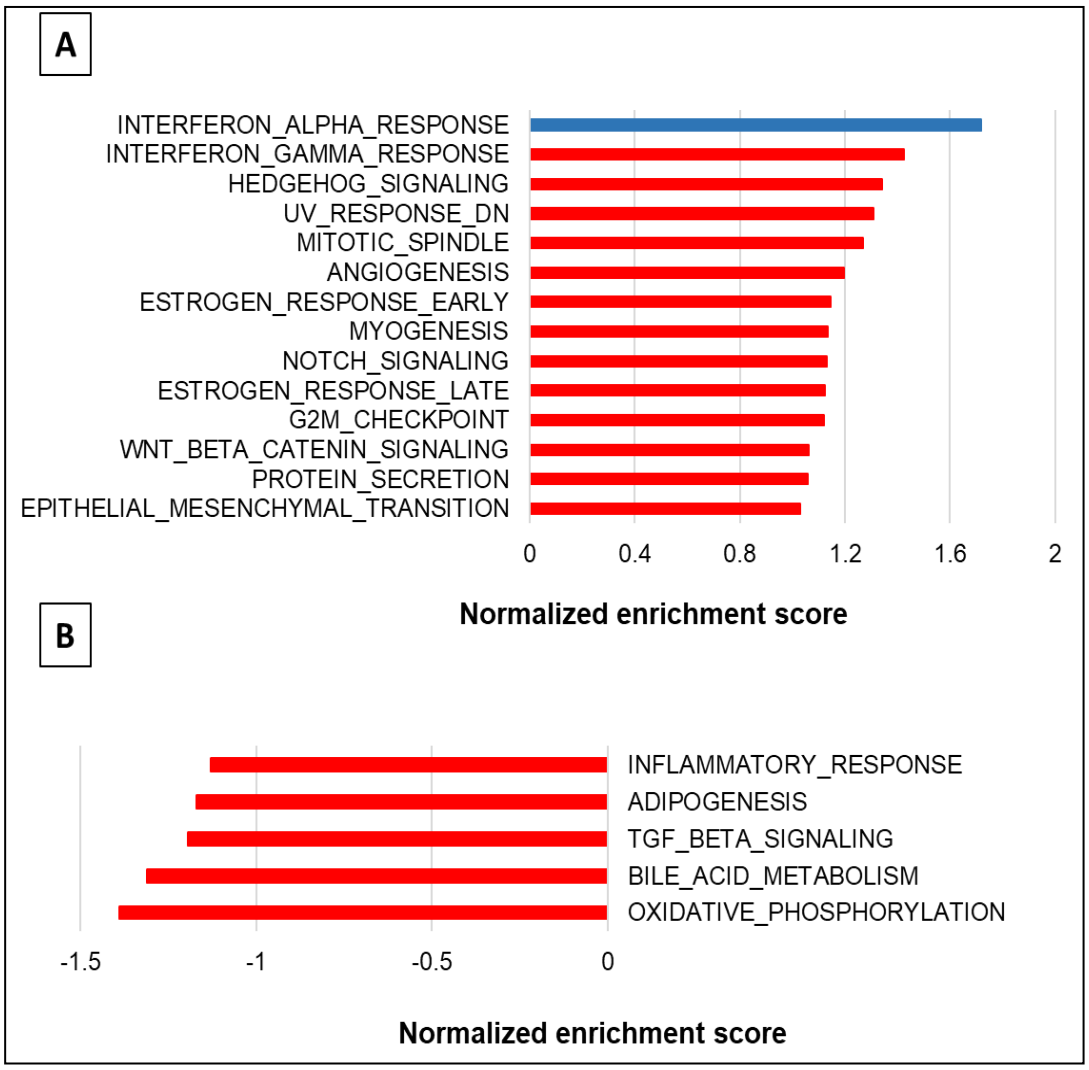


Figure 4. 5: Hallmark pathway analysis of genes expressed by vanillic acid treatment, (A) upregulated and (B) downregulated pathways.

Blue bars represent the significantly changed pathways by vanillic acid in comparison with control group at adjusted *p*-value ≤ 0.05; red bars represent the significantly changed pathways at adjusted *p*-value ≤ 0.7.

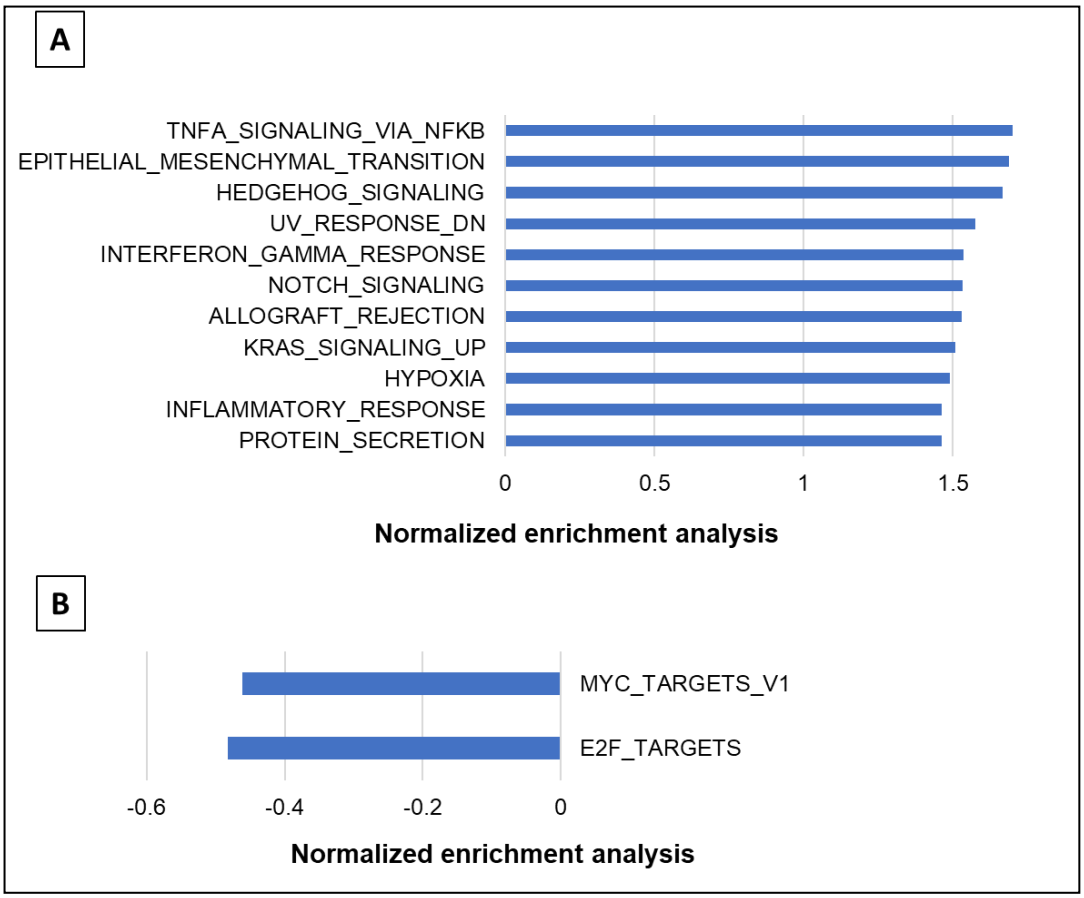


Figure 4. 6: Hallmark pathway analysis of genes expressed by syringic acid treatment, (A) upregulated and (B) downregulated pathways.

Blue bars represent the significantly changed pathways by syringic acid in comparison with control group at adjusted *p*-value ≤ 0.05.

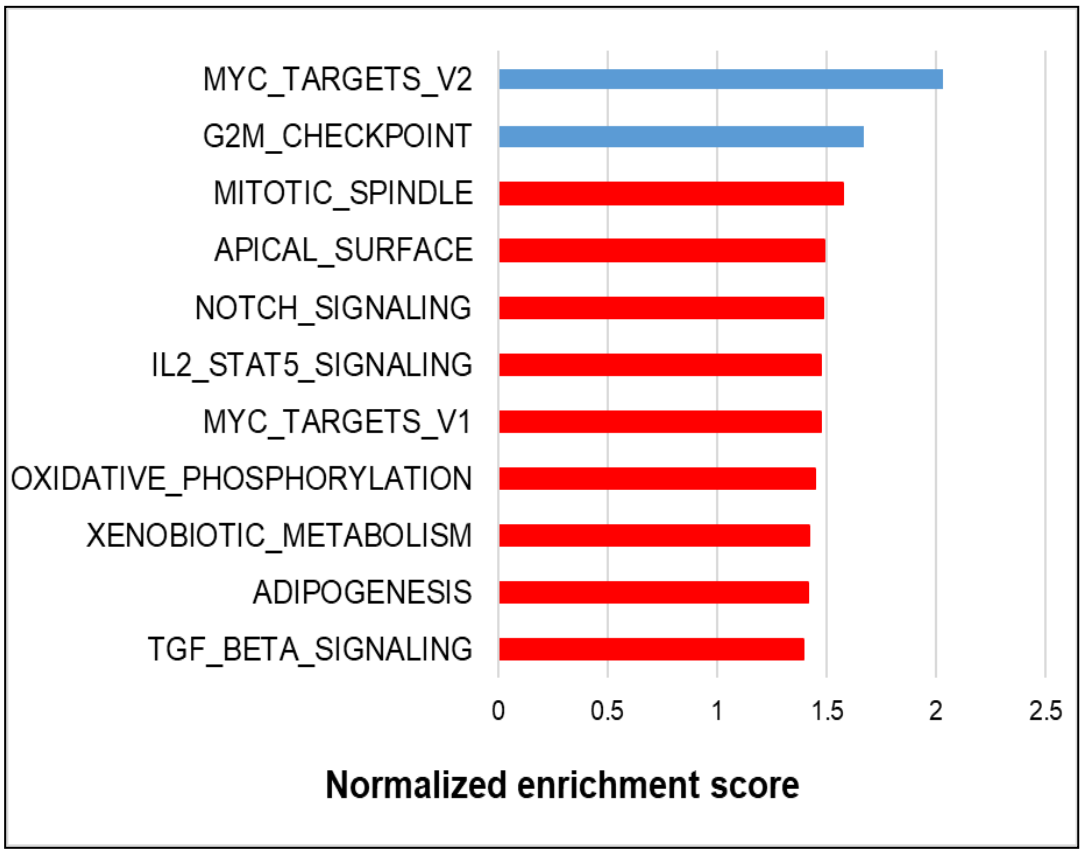


Figure 4. 7: Hallmark pathway analysis of genes expressed by mixture treatment.

Mixture treatment is a mixture of PGA, syringic acid and vanillic acid (3.3 µM each). Blue bars represent the significantly changed pathways by mixture treatment in comparison with control group at adjusted *p*-value ≤ 0.05; red bars represent the significantly changed pathways at adjusted *p*-value ≤ 0.1.

4.6. Discussion

The data presented in this chapter described the RNA transcriptomic study that was conducted as a non-targeted approach to find out which genes involved in atherosclerosis processes are responsive to the major anthocyanin metabolites and to identify any genes and pathways affected by the anthocyanin metabolites (i.e. not usually associated with atherosclerosis) in THP-1 macrophages. The main findings were that (1) no significant changes were observed in the differentially expressed gene expression at adjusted *p*-value ≤ 0.05 to 0.2, (2) the only observation was achieved at adjusted *p*-value ≤ 0.3 and PGA was the only treatment that caused a significant change, (3) most of the 377 affected genes and the enriched pathways after PGA treatment were not atherosclerotic-linked.

The result in the previous two chapters clearly demonstrated that anthocyanins and their metabolites did not affect the expression of the key genes involved in RCT and did not change the biomarkers of HDL function. However, atherosclerosis is a multifactorial disease that can involve many genetic factors and pathways which might have been targeted by anthocyanins. Therefore, the main aim of this chapter was to conduct a transcriptomic study as a non-targeted approach to identify the possible atherosclerotic-related genes and pathways that may be affected by anthocyanins and their metabolites.

mRNA expression profiling using the high-throughput next generation sequencing platforms such RNA sequencing is a new powerful and revolutionary tool for understanding the molecular mechanisms of treatments and discovering new predictive biomarkers, especially in complex disease such as atherosclerosis [334]–[337] .To the best of my knowledge, this study was the first that used RNA sequencing to explore the underlying mechanisms by which anthocyanin reduce the risk factors of CVD. The majority of studies that used non-targeted transcriptomic approaches were conducted using microarray and investigated the effects of anthocyanin-rich fruit or extract [45], [308], [309], [338] without investigating the effects of pure anthocyanin or their metabolites which make the data in this chapter novel.

In this chapter, RNA-seq study was suggested as an additional route to investigate more broadly the effect of anthocyanin metabolites on other atherosclerosis-linked genes and pathways that were not investigated in the previous two chapters and involved in atherosclerosis such as liver X receptor (LXR), retinoic X receptor (RXR), peroxisome proliferator-activated receptors (PPARs), junction adhesion molecules and other members of scavenger receptors family. To test this hypothesis, preloaded

THP-1 macrophages were treated with three different type of anthocyanin metabolites; PGA, vanillic acid, syringic acid and a mixture of them at physiologically relevant concentrations. These metabolites were selected to represent the main metabolites of the predominant type of anthocyanins (C3G and D3G). Syringic acid was the main delphinidin-type metabolites and represents the metabolites of tri-hydroxylated B-ring anthocyanin, while, vanillic acid was the main metabolites of C3G and represent the metabolites of di-hydroxylated-B ring anthocyanins [91], [73]. PGA, on the other hand, represent the metabolites of A-ring structure of C3G and D3G [73], [91]. The reason of choosing these metabolites was to represent the major metabolites of the major three different chemical structure of anthocyanins found in nature, although, there are many others that might be more beneficial and should be investigated in future work.

The result in this chapter support the null hypothesis as there were not any changes in the differentially expressed genes after any of the anthocyanin metabolites treatment. When the adjusted *p*-value cut-off was increased to 0.3, PGA was the only treatment that caused substantial changes in the gene expression. However, the change was not big as the average fold change of upregulated genes was 1.3 ± 0.2, while, the average fold change of downregulated genes was 0.7 ± 0.22 which indicates that anthocyanin metabolites have no substantial effect on atherosclerotic-related genes in THP-1 macrophages. Among those changed genes, CD36 and Plin2 were atherosclerotic-linked genes. CD36 is a member of scavenger receptor B family responsible for binding modified LDL. Previously, it has been reported that downregulation of CD36 caused a reduction in foam cell formation [225]. In contrast to what was expected, PGA upregulated CD36. But the effect was very low and at high adjusted *p*-value which means the upregulation was likely physiologically unimportant. In addition, the RT-qPCR data presented in chapter two support that the effect of PGA in upregulation CD36 was not real as no significant changes were observed in CD36 gene expression with PGA or the other tested metabolites treatments (see section 2.5.3). Similar to CD36, Plin2 gene is involved in lipid droplet formation and foam cell formation. The overexpression of Plin2 increases the lipid accumulation in THP-1 macrophages and hinders cholesterol efflux from macrophages [339]. Although, Plin2 gene expression increased after PGA treatment, the increase was very small which likely lack clinical importance.

In addition to the changes at level of single genes, anthocyanin metabolites showed no substantial effect on the atherosclerotic-linked pathways. Initially, GSEA was conducted using Hallmark database which is one of the most widely used and

comprehensive database [339]. The majority of the changed pathways were not atherosclerotic-linked pathways. Most of them, contrarily, were related to DNA repair and replication such as G2M checkpoint [329], E2 targets [340], androgen response [341], mitotic spindle [342], and hedgehog-signaling [343]. Others were involved in protein secretion and cell prefiltration such as mTORC1 signaling [343], notch-signaling [344]. On the other hand, few dysregulated pathways such as TNFα signaling via NFKB, epithelial-mesenchymal transition, oxidative phosphorylation, interferon gamma response and inflammatory response were linked with inflammation which a key step in atherosclerosis [345]. These pathways could be a target for researcher to further investigate the mechanisms behind the beneficial effects of anthocyanins.

Several studies that used microarray reported that anthocyanin affected genes and pathways that are involved directly in atherosclerosis. Muauray *et al.* (2010) and Mauray *et al.* (2012) found that bilberry anthocyanin-rich extract (0.02 % w/w of standard diet) for two weeks altered the gene expression related to atherosclerosis development in liver and aorta of apo $E^{-/-}$ mice [45], [308]. Moreover, Chen *et al.* (2008) found similar results in mice macrophages with bilberry extract [309]. Additionally, Lefevre *et al.* (2008) reported that grape anthocyanins affected several atherosclerotic-related genes in liver and skeletal muscle of C57Bl/6 mice [338] which was inconsistent with the current data. Probably, the contradiction between this study and the other is due to the difference in the tested material. In the current study, purified anthocyanin metabolites were used, while, anthocyanin-rich fruit or extract were used in the previous reports. The extracts contain many types of anthocyanins that may synergistically affect the genes expression which was not the case in the present study. Another explanation of the lack of effect could be that the most beneficial metabolites haven not been tested as only three metabolites were examined and many other still need to be investigated individually and as mixtures. Additionally, in RNA seq, the number of biological replicates has a large impact on the power of differential expression analysis. The more biological replicates are analysed, the more transcriptional noise can be eliminated and the more clear image and clear differences can be obtained [346]–[348]. Schofield *et al.* (2016), suggested that six replicates should be used in differential expression analysis [349]. In the present study, due to the cost and the number of treatments, four biological replicates were analysed for each treatment which may explain why significant differentially expressed genes was obtained at high adjusted *p*-value and therefore, future work is required with more biological replicates. Furthermore, anthocyanin and their

metabolites may target different cells that are involved in atherosclerosis pathogenesis such as endothelial cells rather than cholesterol-laden macrophages. Krga *et al.* (2018) found that the anthocyanins and their gut metabolites downregulated the monocyte adhesion and trans-endothelial migration pathways in HUVECs cell line using miRNA microarray [249]. Since the project focused on the role of anthocyanin in modulating the lipid metabolism as a mechanism by which anthocyanin attenuate the progression of atherosclerosis, preloaded-THP-1 macrophages was chosen as a model in this experiment to mimic in vivo situation and to be in agreement with last two result chapters that focused on RCT and lipoprotein function. However, the suitability of other cell models is not excluded (see section 2.6). Therefore, additional transcriptomic studies using different cell lines such as endothelial cell lines or liver cell lines are still required to explore the underlying mechanisms of protective effects of anthocyanins.

Overall, there were not any substantial changes observed in the differential gene expression or in the enriched functional pathways after treatments. PGA, on the other hand, was the only treatment that caused relatively big effects, although, the adjusted p-value was high, and the changed genes and pathways were not atherosclerotic-relevant. These findings were consistent with the *in vitro* data that presented in last two chapters in which no significant changes were observed after anthocyanins and their metabolites treatments.

Although, the *in vitro* models are useful for the mechanistic studies, they only allow studying the direct effect in cell levels and do not allow studying the secondary effects or the effects in the organs level and thereby, studying the effects of anthocyanins using an *in vivo* model is necessarily. Since only *in vitro* models were used in the investigation in the last three chapters, using an *in vivo* model was suggested to further investigate the effects of anthocyanin on markers of CVD. Therefore, a randomized controlled trial was conducted to investigate the effects of anthocyanin consumption on lipid/lipoprotein profiles and biomarkers of HDL function.

4.7. Conclusion

In conclusion, the data presented in this chapter showed that the three tested metabolites of anthocyanins did not cause substantial changes in expression of genes and pathways related to atherosclerosis in cultured human macrophages. However, other untested metabolites might be more biologically active or target different cell line rather than cholesterol-laden macrophages. Therefore, future works are still required to investigate the effects of more untested metabolites on transcriptomes in several cell lines. Due to the limitation in the *in vitro* models, an *in vivo* model is required to investigate the secondary effects of anthocyanins and the effects on the body as whole. Therefore, the next chapter will explore the effects of anthocyanins in randomized controlled trial.

CHAPTER FIVE

Chapter five: The effects of consumption of cyanidin-type anthocyanins and delphinidin-type anthocyanins on lipid/lipoprotein profiles and markers of HDL function in humans

5.1. Abstract

Elevated levels of cholesterol and LDL are one of the major risk factors for CVD. On the other hand, HDL is known to protect against atherosclerosis. Not only HDL levels are important, its function and quality are also important. Several human interventions have shown that the consumption of anthocyanin-rich foods and extracts have a beneficial effect on lipid profile and other markers of CVD. However, the beneficial effect of anthocyanins on lipid profile remains inconclusive due to the limitations applied in these studies. Moreover, little is known about the interactions between anthocyanins and biomarkers of HDL function including PON1 genotype. Therefore, the aim of the BERI randomized controlled trial was to compare the effects of cyanidin- and delphinidin-type anthocyanins (two major dietary forms) on lipid/lipoprotein profiles and markers of HDL function. In a double-blind, placebo-controlled, crossover trial, fifty-two hypercholesteraemic males and females (aged ≥ 45 years) with cholesterol higher than 5 mmol/L consumed capsules containing either bilberry extract (delphinidin-type anthocyanins) or black rice extract (cyanidin-type anthocyanins) that provided 320 mg anthocyanins/day for 28 day followed by a minimum 4-week washout period. Lipid/lipoprotein profiles, biomarkers of glycemic control and markers of HDL function were assessed in serum at the start and end of each treatment. All participants were genotyped for Q192R and L55M PON1 polymorphisms. No significant changes were observed in total, LDL- and HDL-cholesterol, triglycerides, cholesterol-HDL ratio, glucose, fructosamine, Apo A1, Apo B, ApoA1-ApoB ratio, HDL-ApoA1 ratio, HDL3 subfraction, HDL2 subfraction, PON1 arylesterase and PON1 lactonase activities. In addition, there was no significant interaction between the participants PON1 genotype, anthocyanin treatments and any of the tested biomarkers of HDL function. These data do not support the notion that consumption of anthocyanins favorably affects circulating lipoprotein profiles, nor do they provide any evidence of interactions with HDL components or HDL function. The lack of effects may be due to the short duration of the intervention.

5.2. Introduction

In the general introduction to this thesis (chapter 1), it was noted that epidemiological studies and meta-analysis of randomized control trial consistently reported a strong association between increased consumption of anthocyanins and reduced CVD risk. It was also noted that several reports of randomized controlled trials suggested that consumption of anthocyanins has protective effects on biomarkers related to CVD, with favourable improvements in lipid profiles being common across many of these studies. In their systematic review, Wallace *et al.* (2016) suggested that anthocyanins have the potential to improve lipid profiles in general, and decrease LDL levels in particular [164]. In another systematic review and meta-analysis of 17 randomized clinical trials that published recently, it was reported that crude and purified anthocyanins consumption positively influenced the lipid profile and inflammatory markers related to CVD [127]. For more details see sections 1.5.2, 1.5.3 and 1.5.4. Additionally, there were another ten recently published papers that reported effects of anthocyanin interventions and improvements in lipid profiles were observed in three of them [132], [140], [144]. However, there were inconsistencies and limitations in most of the randomized controlled trials which make it difficult to reach conclusions with respect the beneficial effects of anthocyanins.

One of the most critical limitations in most of these studies was the lack of an appropriate control [42], [174]–[181]. A Control group should be as identical as possible to the investigational group, so the treatment is the only difference between them. In many previous clinical trials, the placebo control group was absent and the comparison was conducted between the baseline and the endpoint values, or the comparison was conducted between an unhealthy investigational group with a healthy control group which is completely different physiologically to the treatment group [178]. Such limitations make it impossible to conclude whether the effect was from the treatment or a result of confounding factors. Therefore, more rigorous clinical trials with proper controls are still required.

Another important limitation was using whole fruit and vegetables or extracts that contain many bioactives such as vitamins, minerals and other phenolic compounds in addition to anthocyanins. It is difficult to conclude whether the effect is mediated by anthocyanins alone or is due to interactions between several or all of these bioactives. However, sometimes these limitations are ignored when reports of studies with anthocyanins are published. For example, the paper of Kianbakht *et al.* (2014) in which they reported a 27% reduction in cholesterol in humans following 2 months

consumption of whortleberry powder, the authors concluded that this was an effect caused by the whortleberry anthocyanins. However, the anthocyanin concentration in whortleberry is less than 1% (w/w). In fact, the anthocyanin dose in this trial was less than 7.5 mg/day which makes the conclusion that anthocyanins caused the response in cholesterol reduction highly uncertain [162]. Therefore, conducting more trials with purified anthocyanins is fundamental to fill this gap in knowledge. Several studies have used Medox™ which is a mixture of purified anthocyanins from bilberries and blackcurrants [136], [165], [167], [170]–[173][136], [165], [167], [170]. However, Medox™ contains 17 different types of anthocyanin which doesn't allow a comparison between the effects of certain type of anthocyanin. Should a comparison between the effects of different type of anthocyanin help to identify the most beneficial anthocyanin type and define a specific dietary recommendation for health promotion. Therefore, conducting a trial using purified anthocyanin extracts that contain one type or a single group of anthocyanins is necessary to provide information about the biological activity/clinical benefits of specific anthocyanins that can be obtained from specific foods.

Additionally, in chapter two, three and four in this thesis, anthocyanins and their metabolites showed no significant effects on several biomarkers related to RCT and HDL function. However, *in vitro* models were used in this investigation. Although, *in vitro* models are very useful to identify specific molecular mechanisms and provide a means to understand the influence of anthocyanin treatments, *in vitro* models can only show the response in one specific cell type and do not allow studying the secondary effects or investigating the response of the body as a whole. *In vivo* studies, on the other hand, allow investigations that include any secondary effects and to study the responses in organs and tissues level especially in complex disease such as atherosclerosis that involve different organs and tissues. Therefore, conducting a randomized control trial is essential to translate the *in vitro* findings into the *in vivo* situation.

5.3. Objectives

In this chapter, the design, implementation and results from a human dietary intervention study to investigate the effects of anthocyanins on lipid/lipoprotein profiles and HDL quality are reported. The aims of the human randomized controlled study were to (1) compare between cyanidin-type and delphinidin-type anthocyanins in their ability to improve lipid/lipoprotein profiles and markers of glycaemic control and HDL function in humans; (2) investigate the effect of anthocyanin consumption on PON1 arylesterase and lactonase activities; (3) study the association between PON1 genotype, PON1 activities and biomarkers of CVD risk and (4) explore interactions between PON1 genotype, anthocyanin treatments and PON1 activities.

5.4. Materials and methods

5.4.1. Quantification of anthocyanins and other phenolic compounds in bilberry and black rice extracts.

Bilberry extract (*Myrti*PRO®) and black rice extract powders were supplied by the Beijing Gingko Group (BGG), China. According to the manufacturer details, the anthocyanins in bilberry fruit and black rice were extracted with ethanol/water and then purified using a specific column. The purified eluants were then dried to get anthocyanin-rich extract powders. The BGG product specification sheets for both types of extract are provided (Appendix 6). Bilberry extract and black rice extract were analysed for anthocyanin (aglycons and glycosides) and non-anthocyanins phenolics content and composition using an HPLC/MS based method (see section 5.4.1.1 – 5.4.1.3).

5.4.1.1. Extraction

Extraction of anthocyanins were carried out using acidified aqueous methanol (water: methanol: formic acid 70: 28: 2) as described elsewhere [350], [351]. The samples (in triplicates) were rigorously vortexed with solvent for 1 min for homogenization before centrifugation at 13000 rpm for 5 min. The pellet was reextracted three times with the same procedure. All supernatants were pooled and centrifuged before injection on to the HPLC column [350].

For non-anthocyanin phenolics, the extraction was performed using 70% methanol (60°C) and heating for 30 min at 60°C as described previously [352]. The extract was then centrifuged for 5 min at 13000 rpm. The pellet was reextracted three times using

the same procedure and the supernatants were pooled together and centrifuged before injection to HPLC.

5.4.1.2. HPLC/MS analysis

Anthocyanins (aglycons and glycosides) in bilberry and black rice extracts were analysed by reverse phase HPLC/MS using protocol developed in house. 20 µl of sample was injected to Kinetex XB-C18 column (100× 4.6 mm; particle size 2.6 µm) at 40°C. The separation method was carried out using Agilent 1100 series system (HP1100 Agilent Technologies, Waldbronn, Germany) equipped with G1956B single quadrupole mass spectrometer. The mobile phase consisted of 5% aqueous formic acid (eluent A) and 5% formic acid in acetonitrile (eluent B). Samples were eluted with a gradient consisted of 5% B at 0 min, 7% B at 10 min, 10% B at 15 min, 13% B at 16:50 min, 20% B at 18 min, 5% B at 20 min to 25 min and flow rate was 1 ml per min. The anthocyanins were detected by diode array detection (DAD) and the detection was established at 520 nm [59], [353].

Phenolics and flavonoids were analysed similarly with differences in column and conditions as previously described [354], [355]. The samples injected to a Luna C18 column (250 x 4.5 mm 2, 5 µM particle size) (Phenomenex, Macclesfield, UK) and eluted with a mobile phase consisting of 0.1% aqueous formic acid (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The HPLC column temperature was set at 40 °C. The flow rate was 1 ml/min and a gradient consisting of gradient 0% at 0 min, 17% B at 15 min, 25% B at 22 min, 35% B at 30 min, 50% B at 35 min, 100% B at 40 min, 0% B at 55 to 65 min. DAD was used for phenolics and flavonoids detection at 270, 325 and 370 nm.

Procyanidin was analysed as described previously[355], [356]. Briefly, 2 µl of extract was injected to Phenomenex Luna Hilic (150 x 2.0 mm; particle size 3 µm) at 35°C using 2% acetic acid in acetonitrile (eluent A) and 2% acetic acid, 95% methanol and 3% water as eluent B. The flow rate was 0.350 µl/min with an increasing gradient of 7% B at 0 min, 30% B at 15 min, 49% B at 40 min, 7% B at 40:10 to 45 min. Fluorescence detection was achieved using wavelengths 321 nm for emission and 230 nm for excitation.

5.4.1.3. Quantification and qualification

The identification and peak assignment of anthocyanins and non-anthocyanin phenolics in bilberry extract and black rice extract powders were established by running standards for each compound of interest and comparing the retention times

and mass spectral data of each compound with reference standards and previously published data [39], [45], [56], [58]–[60], [233], [357]–[359]. C3G, D3G and malvidin-3-glucoside (Mal3G) (Extrasynthese) were used as standards for anthocyanins quantification and qualification, while, gallic acid, caffeic acid, chlorogenic acid, catechin, epicatechin, phloridzin, myricetin, quercetin, quercetin-3-glucoside and quercetin-3-rhamnoside (Sigma Aldrich, Alfa Aesar, Extrasynthese) were used for quantification and qualification of other non-anthocyanins phenolics. Procyanidin content was calculated using epicatechin as reference standard and the relative response factor that was obtained from apple procyanidns standards [356].

5.4.2. Human intervention (BERI study)

5.4.2.1. Ethics

The protocol was approved by both the Human Research Governance Committee of the Quadram Institute Bioscience and the East of England Cambridge and Hertfordshire Research Ethics Committee (17/WM/0154). The trial was conducted in the Human Nutrition Unit at the Quadram Institute Bioscience, Norwich, UK from September 2017 to August 2018 in compliance with the guidelines laid down in the Declaration of Helsinki. The trial is registered on a publicly-accessible database (NCT03213288). All participants gave written informed consent before participating in the study. The study protocol was written by Wendy Hollands and Paul Kroon at QIB and the recruitment process was managed by Wendy Hollands. My role was mainly helping in sample processing, dealing with participants and conducting the assays.

5.4.2.2. Study population

A total of fifty-two males and females aged ≥ 45 years with a total cholesterol concentration ≥ 5.0 mmol/L at eligibility screening were recruited in and around Norwich, UK from (i) the volunteer database held at the Human Nutrition Unit (HNU) at Quadram Institute Bioscience; (ii) GP surgeries with the help of the co-ordinators from the Primary Research Network (PCRN) – East of England and (iii) from poster and e-mail advertisements placed on the Norwich Research Park and other suitable places. A letter of invitation (Appendix 5) along with a participant information sheet (Appendix 4) were sent to volunteers in the data bases and to those responded to the advertisements. The potential subjects were then screened for eligibility by the HNU research nurse following a 10 hour overnight fast and a blood sample was collected. The subjects were then included/excluded based on the inclusion/exclusion criteria, eligibility questionnaire and the results of clinical laboratory tests. The exclusion

criteria were as follows: total cholesterol level lower than 5 mmol/L; men and women ≤ 45 years of age; smoker; medical condition that judged to affect the outcome such as diabetes; prescribed and non-prescribed medication that affect the study outcome such as statin; depressed or elevated blood pressure measurements (<90/50 or 95/55 if symptomatic or ≥160/100 mmHg); BMI (Kg/m^2) <19.5 or > 40; unwillingness to discontinue dietary supplements 4 weeks prior and for the during the trial; using food for lowering cholesterol such as Benecol or Flora Active; gastrointestinal problem or recent use of colonic irrigation or other bowel cleansing techniques; intend to change their normal use of pre or probiotics during the study; has given blood or intends to donate blood within 16 weeks prior to or during the intervention and clinical results deemed by the HNU Medical Advisor to affect the trial outcome or be indicative of a health problem.

5.4.2.3. Study design

The study was conducted as a randomised, double-blind, placebo-controlled three-arm cross-over trial studying the effects of 28 days consumption of two types of anthocyanin-rich extract on biomarkers for CVD. The three treatments were as follows (i) bilberry extract providing 320mg/day anthocyanins mainly trihydroxylated-B ring type; (ii) black rice extract delivering 320 mg/day anthocyanins mainly dihydroxylated-B ring type and (iii) a placebo control (microcrystalline cellulose). The capsules were opaque, cellulose-based, suitable for oral consumption, had an identical appearance and release their contents within 15 min of reaching the stomach (K-caps vegetarian capsules; GoCap). Each treatment was allocated a letter (A, B or C) by a member of staff not involved with the trial. The randomization sequences list for treatment order were generated using computer-generated block randomization (randomization.com). Each time a participant is successfully recruited onto the study, the order of intervention will be assigned by allocating the participant to the next sequence of letters from the list generated previously. Both researchers, HNU research nurses and the participants will be blinded to the treatments.

The subjects were asked to restrict their intake of berry fruits to a combined maximum of 3 portions per week for 2 weeks before the start of each treatment period and for the 28-d treatment period, including the washout period (4 weeks). In addition, they were asked to exclude completely berry fruits from their diet for the 24 hr preceding test days at the start and end of each treatment period. A list of restricted fruits was provided to each participant (Appendix 3). The participants were asked to fast overnight for a minimum 10 hours with drinking water before undergoing the baseline and post intervention assessment. At the day 1, the baseline assessment was

conducted, and a 35 ml of blood was collected from fasted participants. Participants then consumed 4 capsules (equivalent to the daily dose) with plenty of water once daily in the morning for 28 days with a minimum 4 weeks washout period. To assist with compliance to treatment, participants were provided with a capsule checklist and asked to record the consumption of the capsules on each day and return the unused capsules and the record sheet at the end of the treatment period. Participants were also contacted 2-weeks into each of the treatment periods to assess progress. The compliance was assessed using the record sheet and by counting the unused capsules returned at the end of 28 days of treatment. Participants who ingest less than 80% of the capsules over the entire treatment period may be withdrawn from the study. The compliance with dietary restriction was assessed measuring the anthocyanins and their metabolites in urine at the baseline. On day 29, a further 35 ml of blood was obtained from fasted participants. A minimum of 4 weeks washout period was performed before starting the next phase. Fig 5.1 illustrates the study design. The primary outcome measure for this trial was change (0 to 28 days) in LDL-cholesterol, while the secondary outcome measures were the changes in total and HDL-cholesterol, triglycerides, apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), HDL3, HDL2, PON1 activities, glucose and fructosamine**.**

5.4.2.4. Blood processing

The blood was collected from fasted participants at day1 and day 29 and processed as previously described [141]. Briefly, whole blood was collected into serum separating tubes (Becton Dickenson) and allowed to clot for 30 min at room temperature before centrifugation at 2000 x g for 10 mins. Sera were then aliquoted and stored at –80°C. For PON1 genotyping, a sub-sample of whole blood was collected in EDTA tubes and stored at -80°C until used.

5.4.2.5. Biochemical markers for CVD analysis

Total, HDL- and LDL-cholesterol, triglycerides, apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), HDL3, HDL2, glucose and fructosamine were analysed using an automated bench top clinical chemistry analyser (Randox Daytona plus) according to the manufacturer's instructions. The Randox Daytona instrument uses an enzymatic reagent system coupled to a photometric detector system based on a specific analytical method for each compound of interest. LDL, HDL, HDL3 were analysed by a clearance method, total cholesterol and fructosamine were analysed by an enzymatic method, triglycerides and glucose were analysed by a GPO-POD method, while, ApoA1 and ApoB were analysed by an immunoturbidimetric method. HDL2 levels were estimated by subtracting HDL3 from total HDL.

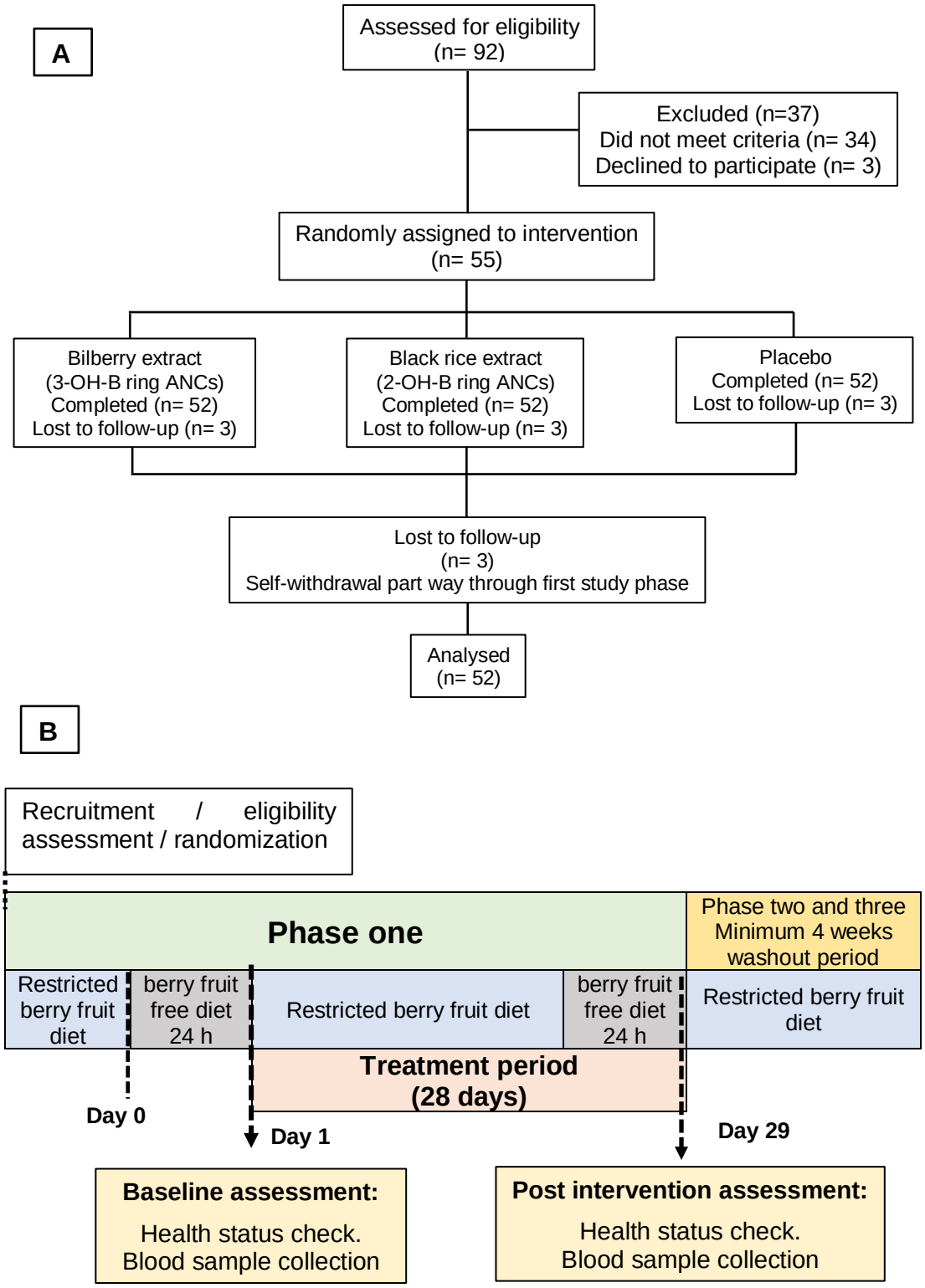


Figure 5. 1: (A) Flow diagram of participants through the trial and (B) Study protocol detailing the involvement of participants through the study.

3-OH-B ring ANCs = trihydroxylated-B ring anthocyanins, 2-OH-B ring ANCs = dihydroxylated-B ring anthocyanins

5.4.2.6. PON1 activities

PON1 arylesterase and lactonase activities were measured as described earlier in sections 3.4.2.1 and 3.4.2.2 with some modifications. Briefly, arylesterase was measured by adding 20 µl of diluted serum (50-fold in assay buffer) to 80 µl assay buffer in 96-well plate. After incubation for 10 min at 37°C,100 µl of *p*-nitrophenyl acetate (2 mM) was add to the previous mixture. Therefore, the final volume was 200 µl and the concentration of substrate was 1 mM, while the final concentration of serum was 0.2%. The increase in absorbance was measured immediately at 410 nm and over 10 min using a FLUROstar Optima plate-reader (BMG labtech, UK). Similarly, lactonase activity was measured by adding 20 µl diluted serum (90-fold dilution in the assay buffer) to 80 µl assay buffer and 50 µl of DTNB (4 mM) in a 96-well plate prior to incubation at 37°C for 10 min. After that, 50 µl of TBBL (2 mM) was added and the absorbance was measured immediately at 412 nm. The final volume was therefore 200 µl and the concentration of reactants were 0.1% serum,1 mM DTNB and 0.5 mM TBBL.

In order to quantify the activities that catalysed only by PON1 (and not other serum arylesterases/lactonases), PON1 lactonases/arylesterase was measured with 100 µM of 2-hydroxyquinoline (2-HQ) alongside the assay without inhibitor. 2-HQ is a selective competitive inhibitor of PON1[235], [254], [294], [360], [361]. PON1-mediated arylesterase/lactonase activity was then calculated by subtracting the activities in the presence of 2-HQ from the total arylesterase/lactonase in the absence of 2-HQ (Fig 5.2). Each 96-well plate included blank control samples (no serum) to correct for any spontaneous non-enzymatic hydrolysis of substrates. The assays were performed in duplicate for each sample and the average measurement was calculated. A standard curve of p-nitrophenol (arylesterase product) was performed under the same conditions to quantify the enzyme units of arylesterase, while the extinction coefficient of TBBL ($\varepsilon_{412\ nm}$ = 7000 $OD.M^{-1}.cm^{-1}$) value was used to calculate the enzyme units for lactonase. One unit of arylesterase activity is equal to 1 µmol of phenyl acetate hydrolysed per ml per minute, while, enzyme unit of lactonase activity is equal to 1 µmol of TBBL hydrolysed per ml per minute.

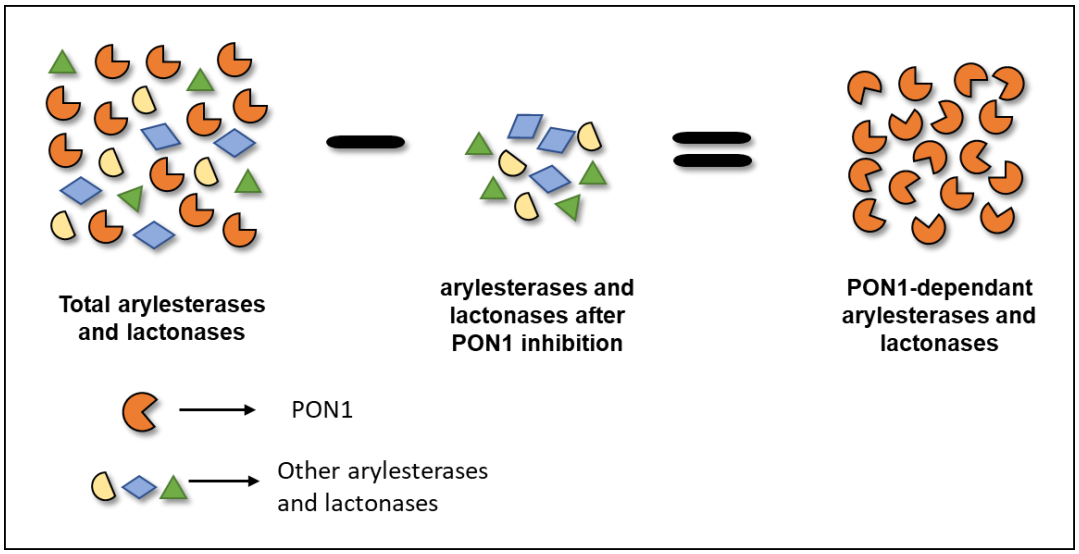


Figure 5. 2: An illustration of the measurement of PON1-mediated activities.

Arylesterase/lactonase activity was measured with and without a PON1 inhibitor. PON1-dependant arylesterase/lactonase activity was measured by the subtraction of the activities in the presence of 2-HQ from the total arylesterase/lactonase in the absence of 2-HQ.

5.4.2.7. PON1 genotypes

All participants were genotyped for their Q192R and L55/M polymorphisms using PCR amplification as previously described [362]–[366]. Genotyping by this method is simply conducted using PCR reactions with primers designed to amplify the exon that contains the SNP. The PCR product is then purified and sequenced using standard sequencing techniques. The sequence was then aligned to the reference genome to identify the genotype.

DNA was extracted and purified from whole blood using a DNeasy Blood & Tissue Kit (Qiagen, Cat# 69504) according to the manufacturer's protocol. DNA quantity and quality were measured using the NanoDrop ND-1000 spectrophotometer. The samples were aliquoted and stored at -20°C until using.

To sequence exon 3 (contains L55M SNP) and exon 6 (contains Q192R SNP), specific primers designed for each exon were used in the PCR reactions. The forward and reverse primers were designed using NCBI Reference Sequences and Primer-BLAST. (https://ncbiinsights.ncbi.nlm.nih.gov/). To allow sequencing of the entire exon, the primers were designed to cover small additional sequences from adjacent introns. PCR amplification of exon 3 (L55M polymorphism) was performed using the following primers: Forward 5`-CCCAGTTTCAAGTGAGGTGT-3` and reverse 5`TGGGCATGGGTATACAGAAAGC-3` (PCR product size 240-bp). The primers for amplification of exon 6 (Q192R polymorphism) were: Forward 5`-AGACAGTGAGGAATGCCAGTTA-3` and reverse 5`TCTCCTGAGAATCTGAGTAAATCCA-3` (PCR product size 333-bp).

PCR reactions were performed using a Veriti® 96-Well Thermal Cycler (Applied Biosystems) as described previously in section 2.4.6 with some modifications. The reaction mixture (20 µl) comprised of DNA template (20ng), 1 X ImmoMix PCR MasterMix (Bioline, Cat. # BIO-25022), magnesium chloride (0.5 mM), BSA (50 µg/ml) and 10 nM forward and reverse primers. The following PCR cycling conditions were used; one cycle at 95°C/10 min for initial denaturation, followed by 35 cycles of amplification (95 °C/15 sec, 60 °C/1 min and 72°C/30sec) then a final extension of 7 min at 72°C. To check the product size, samples of amplified products were separated alongside DNA standard ladder in 1.5% agarose gel at 50 V for 1 hour. The bands were visualised using ethidium bromide solution (0.5µg/ml) and the gel then was exposed to UV light and the picture was taken with gel imaging system. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Cat# 28104) according to the manufacturer's protocol. The purified products were aliquoted and stored at -20°C or sent for sequencing (Eurofins, UK). To determine the genotypes, the sequences were aligned to the human reference genome and SNPs were identified using Ugene software for windows version 1.29.0.

5.4.2.8. Statistical analysis and power calculation

The primary outcome measure in this trial was LDL-cholesterol. The study was powered to detect a change of 0.14 mmol/L in LDL- cholesterol (relative to placebo) at a significance level of 0.05 with 90% power. Assuming a standard deviation similar to that observed in the data from Zhu *et al.* 2013 (approximately 0.5 mmol/L) [167], the power calculation indicated that 50 participants were required to complete the trial. The power calculation has performed according to the primary outcome and not for the secondary outcome.

The analysis was performed using the changes in parameters which was calculated by subtracting the measurements at the end of the intervention from the measurements at day 1 (baseline). No baseline correction has been done.

All data and statistics were analysed by using GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). All values are given as means ± SD. Any statistical difference between the study arms was determined with one-way ANOVA coupled with Dunnett's Multiple Comparison Test comparing all study arms with placebo arm. Newman-Keuls' Multiple Comparison Test was used to conduct all possible pairwise comparisons in the case of studying the association of PON1 genotypes and haplotypes; activities. Values of $p \leq 0.05$ were considered significant.

5.5. Results

5.5.1. Identification and quantification of anthocyanins and other (poly)phenolics in bilberry and black rice extracts

Anthocyanins quantity and identity were determined for the two anthocyanin-rich extracts used in the BERI study, bilberry (MyrtiPRO®) and black rice extracts powder, using reverse-phase HPLC analysis with DAD detection at 520 nm. Identification was performed using the mass obtained from a single quadrupole mass spectrometer and matching the mass and retention time with authentic standards and with data reported previously for bilberry and black rice. C3G was used as a reference standard to quantify dihydroxylated-B ring anthocyanins, D3G was used as a reference standard to quantify trihydroxylated-B ring anthocyanins, while, Mal3G was used as a reference standard to quantify malvidin-types of anthocyanins (methylated-B ring anthocyanins).

A total of 14 anthocyanins were identified and quantified in the bilberry extract powder as shown in Fig 5.3. The total anthocyanin content was 436.8 ± 4.6 mg (g powder)$^{-1}$ (Table 5.1). The major anthocyanin types that were identified and quantified in the bilberry extract were delphinidin-3-galactoside (15.1%), delphindin-3-glucoside (15.0%), delphinidin-3-arabinoside (13.9%), whereas, peonidin-3-arabionoside was found to be the least concentrated anthocyanin in this extract. Cyanidin-3-glucoside and cyanidin-3-galactoside were the main dihydroxylated-B ring anthocyanins with content of 9.5 and 9.2% of total anthocyanins in bilberry extract powder, respectively. As shown in Fig 5.3 and Table 5.1, trihydroxylated-B ring anthocyanins (delphinidin, petunidin and malvidin types) were the predominant type of anthocyanins in the bilberry extract powder and they represented approximately 76% of total anthocyanins that were quantified in the bilberry extract. Since malvidin and petunidin are methylated forms of delphinidin, bilberry extract powder can be considered as a good representative of delphinidin type of anthocyanins.

In contrast, only 5 anthocyanin types were identified in black rice extract and all of them were dihydroxylated-B ring anthocyanins (Fig 5.4). The total anthocyanin content was 312.2 ± 1.6 mg (g powder)$^{-1}$ expressed as cyanidin-3-glucoside. About 90% of the total anthocyanins was cyanidin-3-glucoside, while peonidin-3-glucoside content was about 7% and traces of the other dihydroxylated-B ring types (Table 5.2). The content of non-anthocyanins was also analysed for bilberry and black rice powder extracts using reverse-phase HPLC. The total polyphenol content included the following analysed compounds: Gallic acid, caffeic acid, chlorogenic acid, catechin,

epicatechin, phloridzin, myricetin, quercetin, quercetin-3-glucoside and quercetin-3-rhamanoside using authentic standards for these compounds. In bilberry extract powder, non-anthocyanin phenolic content was very low with about 35.1 mg (g powder)$^{-1}$ (3.5% of powder) mainly due to chlorogenic acid. In black rice extract, only traces of non-anthocyanin phenolics were detected but were not quantified as the signals were very low, even though a large quantity of powder was extracted.

Procyanidins were analysed in extracts using normal-phase HPLC combined with a fluorescence detector at wavelengths 230 nm for excitation and 321 nm for emission. Procyanidins were then quantified using the relative response factor that had previously been established for apple procyanidins in our lab and which were published recently [354]. In bilberry extract powder, procyanidins with degree of polymerization (DP) from 2 to 6 were separated and detected. However, the signals were very weak. The total monomeric and oligomeric procyanidins accounted for 27.6 mg (g powder)$^{-1}$ of bilberry extract. Only traces of monomeric procyanidins were detected in black rice extract powder, while oligomeric procyanidins were not detected, even though a high concentration of extract had been used in the analysis.

Based on these results, non-anthocyanins (poly)phenolics were minor components of the bilberry extract and present only in trace quantities in black rice extract. Anthocyanins were the predominant (poly)phenolics found in both extracts. Therefore, any effect of treatments will almost certainly be due to anthocyanins. The composition data confirmed that the bilberry extract and black rice extract were very rich sources and good representative of tri-hydroxylated-B ring (delphinidin type) and dihydroxylated-B ring (cyanidin type) of anthocyanins, respectively.

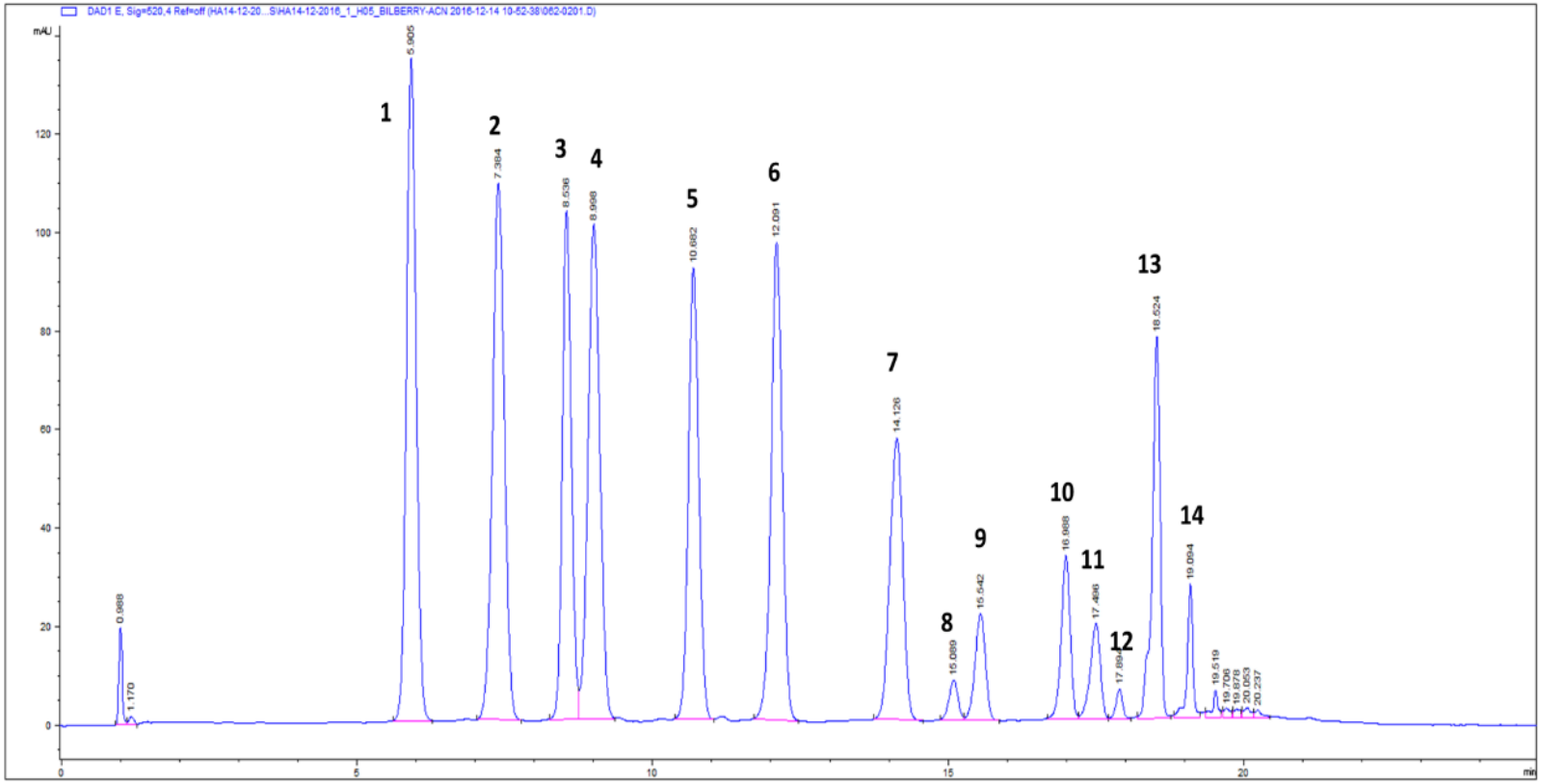


Figure 5. 3: HPLC anthocyanins chromatogram (at 520 nm) of bilberry extract (100 µg/ml).

The extraction was performed in (water: methanol: formic acid 70: 28: 2). The mobile phases were 5% aqueous formic acid (eluent A) and 5% formic acid in acetonitrile (eluent B). The peak number corresponding to anthocyanin name is shown in Table 5.1

Table 5. 1: Anthocyanins profile and content in bilberry extract powder[1]

Peak number	Anthocyanin type	Content mg (g powder)$^{-1}$	% from total anthocyanins[2]
Peak 1	Delphindin-3-galactoside	65.84 ± 0.07	15.07
Peak 2	Delphindin-3-glucoside	65.31 ± 0.10	14.95
Peak 3	Cyanidin-3-galactoside	40.23 ± 0.07	9.21
Peak 4	Delphindin-3-arabinoside	60.66 ± 0.09	13.89
Peak 5	Cyanidin-3-glucoside	41.79 ± 0.05	9.57
Peak 6	Petunidin-3-galactoside	55.27 ± 0.14	12.65
Peak 7	Petunidin-3-glucoside	37.85 ± 0.06	8.66
Peak 8	Peonidin-3-galactoside	3.57 ± 0.02	0.82
Peak 9	Petunidin-3-arabinoside	11.77 ± 0.04	2.69
Peak 10	Peonidin-3-glucoside	13.92 ± 0.05	3.19
Peak 11	Malvidin-3-galactoside	7.77 ± 0.03	1.78
Peak 12	Peonidin-3-arabinoside	2.32 ± 0.03	0.53
Peak 13	Malvidin-3-glucoside	23.18 ± 0.07	5.31
Peak 14	Malvidin-3-arabinoside	7.35 ± 0.13	1.68
	Total	**436.8 ± 0.82**	0.82

[1] Values are mean ± SD.

[2] The percentage was calculated using the average of content of each analyte. Three separate samples of extract and three replicates per mass of extract were analysed.

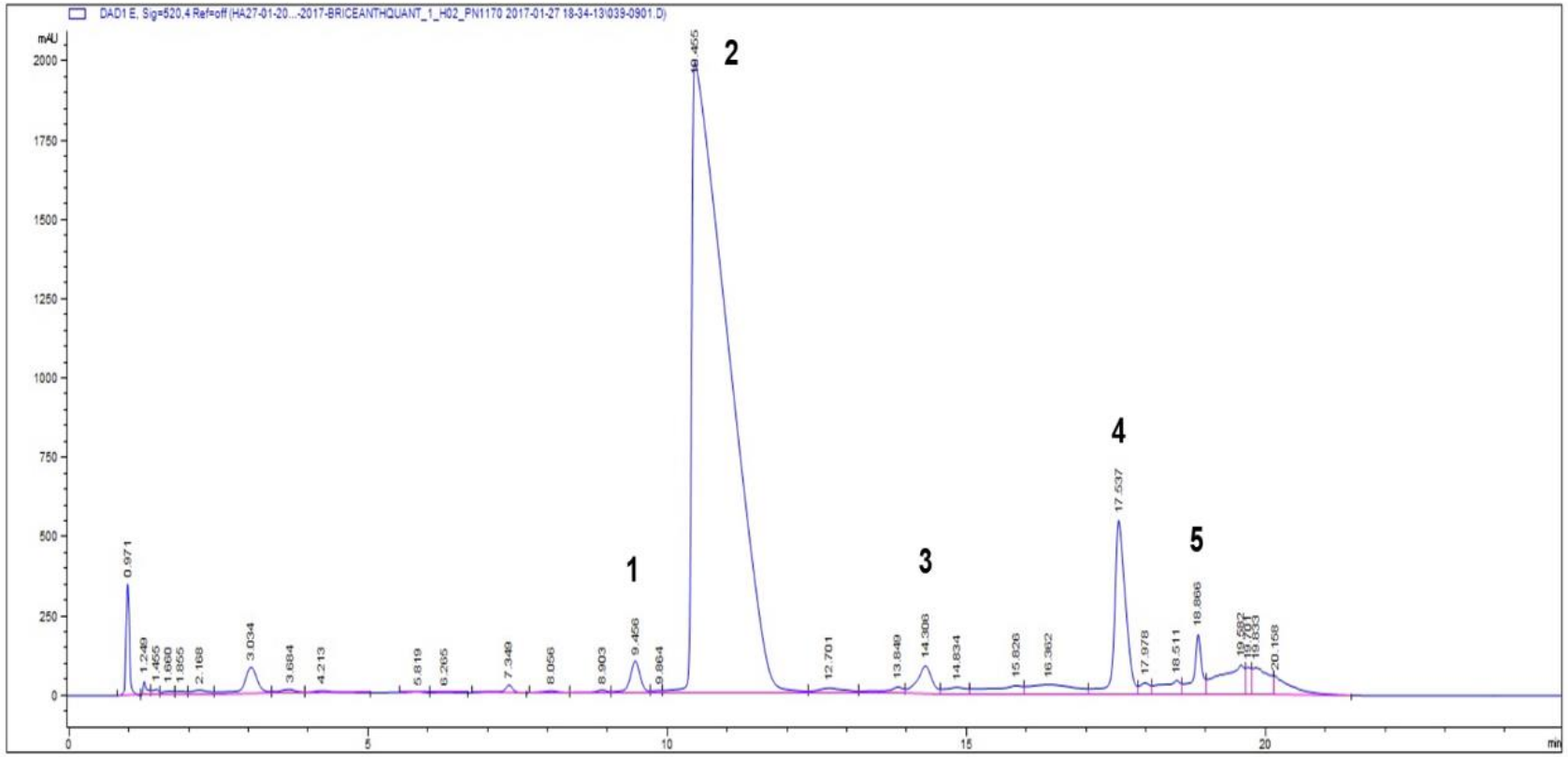


Figure 5. 4: HPLC anthocyanins chromatogram (at 520 nm) of black rice extract (100 µg/ml).

The extraction was performed in (water: methanol: formic acid 70: 28: 2). The mobile phases were 5% aqueous formic acid (eluent A) and 5% formic acid in acetonitrile (eluent B). The peak number corresponding to anthocyanin name is shown in Table 5.2

Table 5. 2: Anthocyanins profile and content in black rice extract powder[1]

Peak number	Anthocyanin type	Content mg (g powder)$^{-1}$	% from total anthocyanins[2]
1	Cyanidin-3,5-diglucoside	3.77 ± 0.01	1.21
2	Cyanidin-3-glucoside	278.61 ± 0.6	89.25
3	Cyanidin-3-*O*-(6''-*O*-*p*-coumaryl) glucoside	4.41 ± 0.01	1.41
4	Peonidin-3-glucoside	22.4 ± 0.11	7.17
5	Peonidin-3-*O*-(6''-*O*-*p*-coumaryl) glucoside	5.94 ± 0.11	1.9
	Total	**312.15 ± 0.75**	**100**

[1] Values are mean ± SD.

[2] The percentage was calculated using the average of content of each analyte. Three separate samples of extract and three replicates per mass of extract were analysed.

5.5.2. Study population and baseline characterization.

Of the fifty-five subjects randomly assigned to treatments, fifty-two participants completed the study (twenty-four men and twenty-eight women). Three participants self-withdrew part way through the first phase, mentioning difficulties with study time commitments as the reason for withdrawal. Overall compliance to treatments was high. When assessed as a proportion of the intended total, > 99% of capsules were ingested across all three treatments. As an indication of excellent compliance, the total anthocyanin was quantified in urine. In the baseline 24-h urine collections, small quantities of anthocyanins were detected and was similar across all three treatment arms (28 ± 36, 23 ± 25 and 30 ± 62 nmols; placebo, bilberry extract and black rice extract, respectively). After 28 days treatment, the quantities of anthocyanins detected in the 24-h urine collections was substantially higher (631 ± 539 and 299 ± 204 nmols; bilberry extract and black rice extract, respectively), while, the amount of anthocyanins in urine after placebo remained unchanged (22 ± 21) indicating excellent compliance with the capsule ingestion regime and that the anthocyanins in treatment were available and can demonstrate a biological effect (the quantification of anthocyanins was performed by Jasmin Perceval, unpublished data).

Fig 5.1 (in material and method section) describes the flow of participants through the study and Table 5.3 shows the baseline demographics and values for biomarkers of CVD at the beginning of the study. The baseline characteristics at eligibility assessment were summarized in Table 5.3. There were no serious adverse events were reported during the study.

5.5.3. Effect of the intervention on lipid profiles

Total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride were quantified in serum before and after 28 days consumption of 320 mg/day of either bilberry extract (delphinidin type of anthocyanins) or black rice extract (cyanidin type of anthocyanins) in 52 hypercholesterolemic individuals using Randox. The change (0 to 28 days) was calculated by subtracting the measurements at the end of the intervention from the measurements at day 1. Firstly, no effects of the treatments was observed for LDL-cholesterol, the primary outcome of the intervention compared to the placebo control group (Fig 5.5). In addition, total cholesterol, HDL-cholesterol and triglyceride remained unchanged (Fig 5.6 to 5.8). Moreover, the treatments had no effect on the cholesterol/HDL ratio (Fig 5.9).

The treatment-placebo average difference in all these lipoprotein biomarkers was close to zero. HDL, triglyceride and cholesterol/HDL ratio values were within the reported physiological ranges. However, cholesterol and LDL-cholesterol were higher than the normal physiological ranges which was expected as we recruited hypercholesterolemic individuals.

Table 5. 3: Baseline demographics and biomarkers of CVD of the subjects at start of trial[1]

Measurement variable	Mean	SD
Age (year)	62.6	7.8
Gender	24 males and 28 females	
BMI (kg/m2)	25.9	3.4
Cholesterol (mmol/L)	6.2	0.9
HDL-C (mmol/L)	1.7	0.5
LDL-C (mmol/L)	3.9	0.8
Triglycerides (mmol/L)	1.3	0.8
Cholesterol/HDL ratio	3.8	1.3
ApoA1 (mg/dL)	229.1	62.6
ApoB (mg/dL)	128.0	25.7
Glucose (mmol/L)	5.5	0.4
Fructoseamine μmol/L	268.8	22.3
HbA1c (mmol/mol)	35.3	3.7
Haemoglobin (g/L)	144	12
Systolic blood pressure (mmHg)	134	15.3
Diastolic blood pressure (mmHg)	79	10.8

[1] Values are mean ± S.D., n=52. BMI= body mass index.

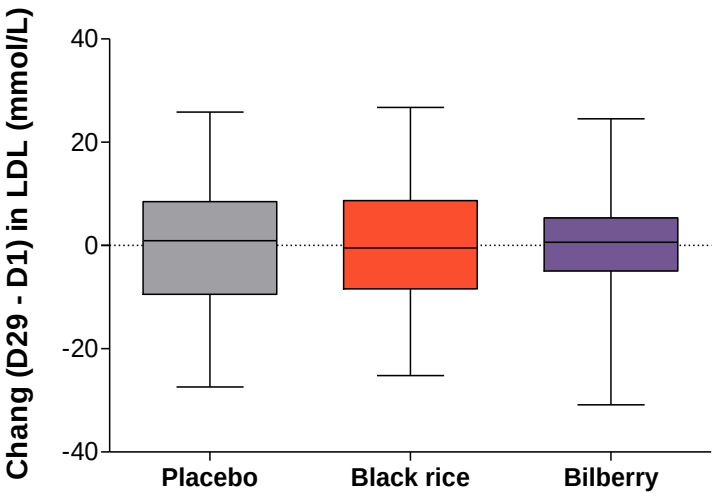


Figure 5. 5: Change in LDL-cholesterol in serum of humans after 28 days dietary intervention.

LDL-cholesterol in serum of all 52 hypercholesterolemic individuals was quantified at the start and end of the intervention with bilberry or black rice extract capsules that provided 320 mg/day anthocyanins. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). One measurement was conducted for each participant.

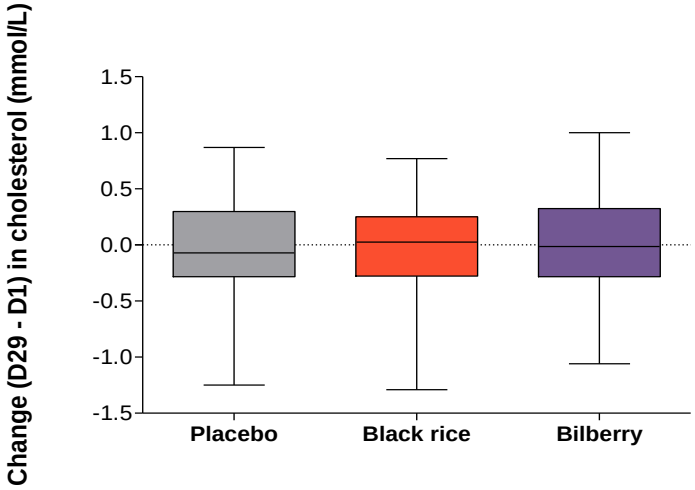


Figure 5. 6: Change in total cholesterol in serum of humans after 28 days dietary intervention.

Total cholesterol in serum of all 52 hypercholesterolemic individuals was quantified at the start and end of the intervention with bilberry or black rice extract capsules that provided 320 mg/day anthocyanins. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). One measurement was conducted for each participant.

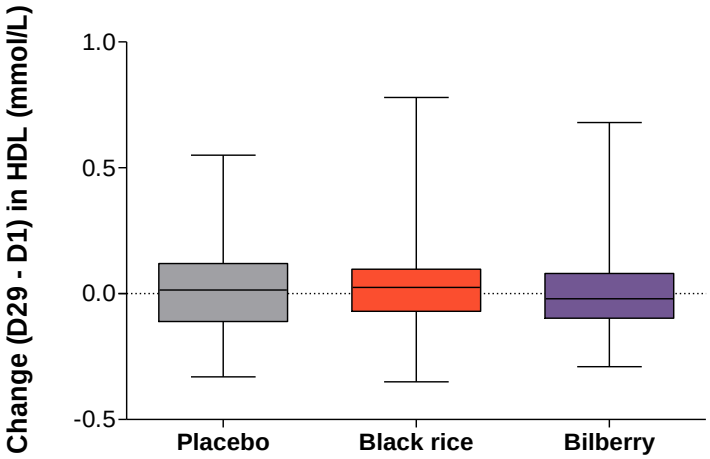


Figure 5. 7: Change in HDL-cholesterol in serum of humans after 28 days dietary intervention.

HDL-cholesterol in serum of all 52 hypercholesterolemic individuals was quantified at the start and end of the intervention with bilberry or black rice extract capsules that provided 320 mg/day anthocyanins. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). One measurement was conducted for each participant.

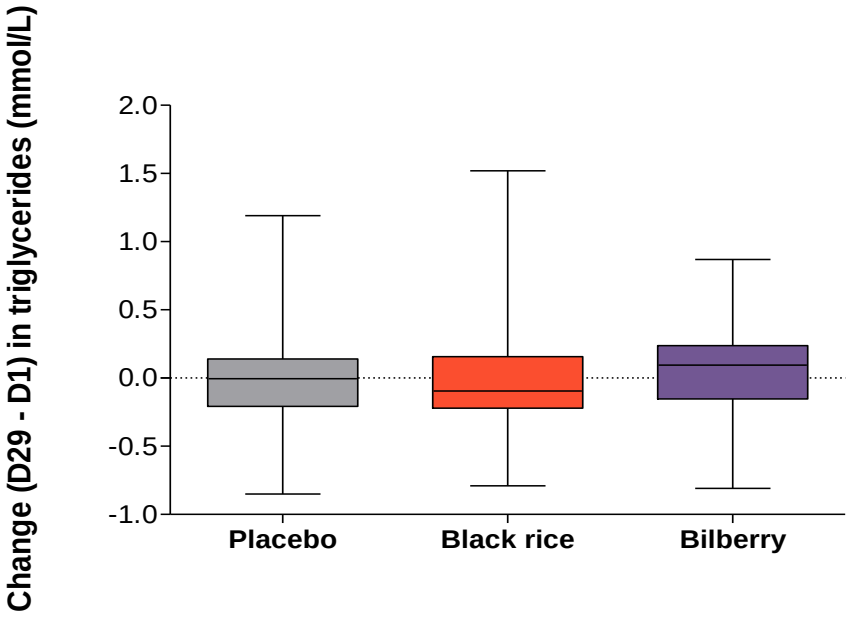


Figure 5. 8: Change in triglycerides in serum of humans after 28 days dietary intervention.

Triglycerides in serum of all 52 hypercholesterolemic individuals was quantified at the start and end of the intervention with bilberry or black rice extract capsules that provided 320 mg/day anthocyanins. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). One measurement was conducted for each participant.

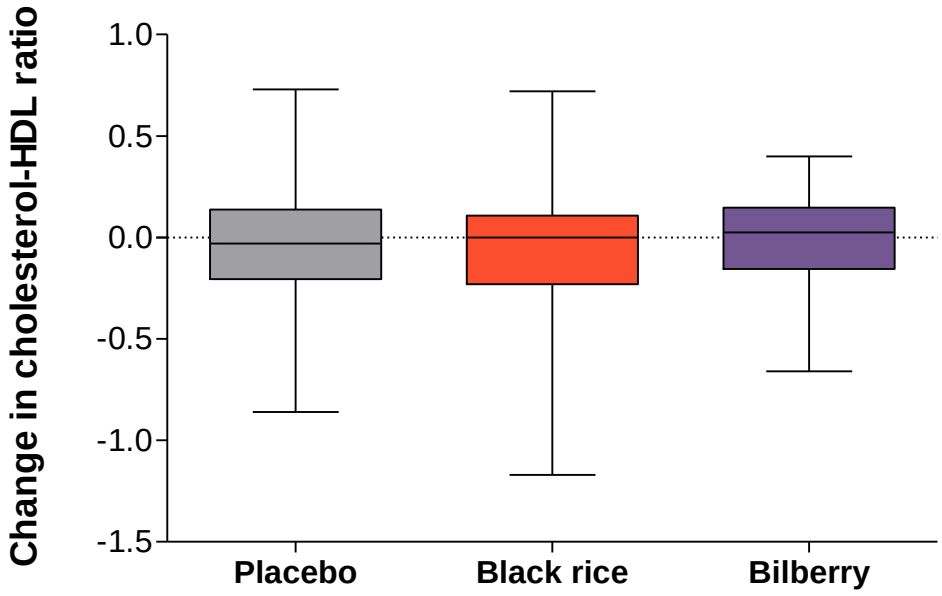


Figure 5. 9: Change in total cholesterol / HDL ratio in humans after 28 days dietary intervention.

Total cholesterol / HDL ratio of all 52 hypercholesterolemic individuals was quantified by dividing the total cholesterol level of each individual by their HDL at the start and end of the intervention with bilberry or black rice extract capsules that provided 320 mg/day anthocyanins. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$).

5.5.4. Effect the treatments on Apo A1, Apo B and HDL subfractions

The circulating levels of Apo A1 (the major protein component of HDL) and Apo B (the major protein component of LDL) were quantified in serum of all 52 hypercholesterolemic individuals at the start and end of the intervention with bilberry and black rice extract using the turbidity method by of the Randox autoanalyser. As shown in Fig 5.10 A and B, the intervention with bilberry extract did not statistically change either Apo A1 or Apo B proteins levels compared to the placebo group, nor did the intervention with black rice extract. The average change values of Apo A1 and Apo B were close to zero (mg/dL) after intervention. Similarly, the HDL-Apo A1 ratio and ApoA1-Apo B ratio did not statistically change after treatments and the average change values were close to zero (Fig 5.11 A and B).

Another common marker in humans for lipid metabolism and HDL function is the profile of HDL subfractions. Therefore, the effect of the treatments on HDL3 and HDL 2 was investigated (Fig 5.12 A and B). In the serum samples of all 52 participants, HDL subfraction (HDL3) was measured using clearance method by Randox at the start and end of the intervention. HDL2 was quantified by calculating the difference between total HDL and HDL3. As observed in the analysis of total HDL, there was no significant differences between study arms in either HDL3 or HDL2 subfractions and the average of changes was close to zero (mmol/L).

5.5.5. Effect of the treatments on biomarkers of glycaemic control

Additional to the quantification of lipid and lipoproteins profile in serum of human subjects, the effect of anthocyanins consumption on glycaemic control was measured. The long-term effect of consumption of anthocyanins on glycaemic control was measured by quantifying glucose and fructosamine levels in serum as alternative biomarkers of glycaemic index at the start and end of the the intervention using Randox. After 28 days consumption of either bilberry or black rice extract, no significant differences between treatments and control group were observed in either glucose or fructosamine (Fig 5.13 A and B). The change in glucose and fructosamine was close to zero (mmol/L and μmol/L, respectively) in placebo, black rice and bilberry groups. All values were within the normal reported physiological ranges (3.9 - 7.1 mmol/L for glucose and 258 - 317 μmol/L for fructosamine).

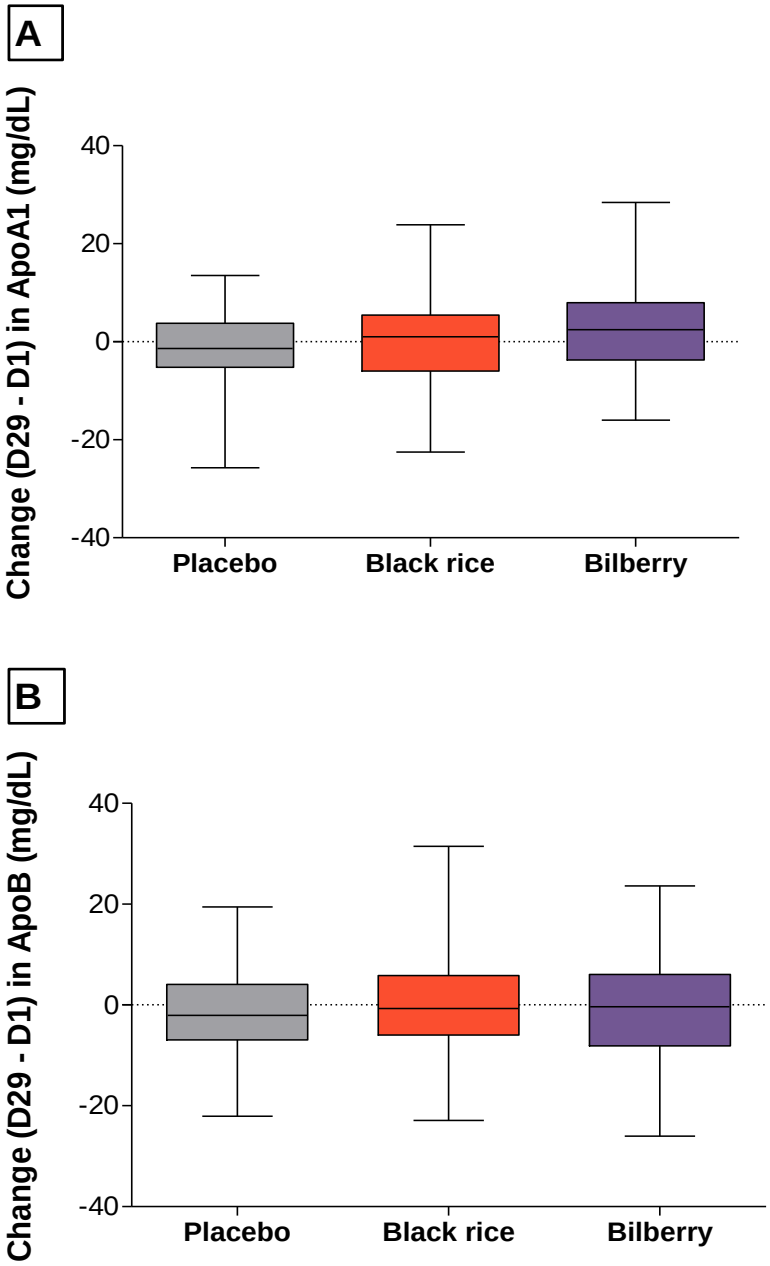


Figure 5. 10: Change in (A) Apo A1 protein levels and (B) Apo B protein level in serum of humans after 28 days dietary intervention.

Apo A1 and Apo B levels in serum of all 52 hypercholesterolemic individuals were quantified at the start and end of the intervention with bilberry or black rice extract capsules that provided 320 mg/day anthocyanins. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). One measurement was conducted for each participant.

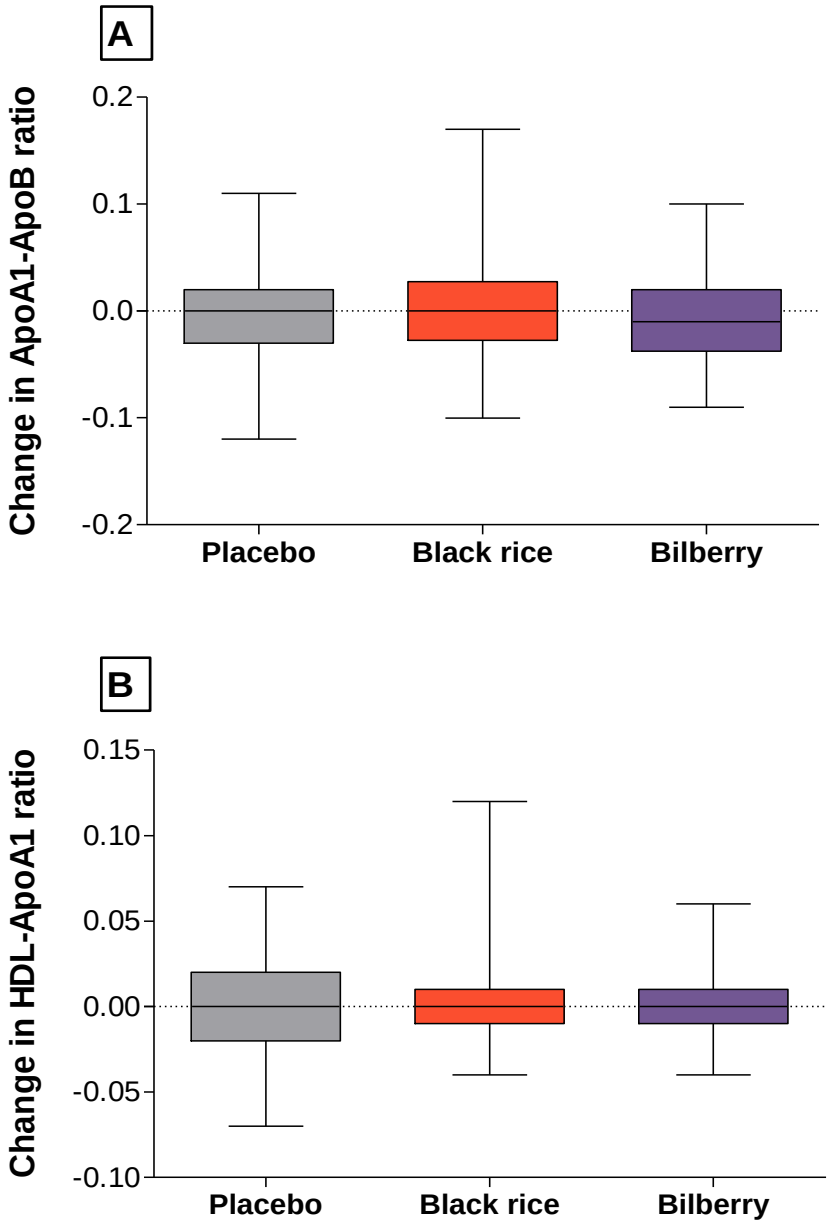


Figure 5. 11: Change in (A) HDL-ApoA1 ratio and (B) ApoA1-ApoB ratio in serum of humans after 28 days dietary intervention.

Data are shown as means ± SD (n=52). No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). One measurement was conducted for each participant.

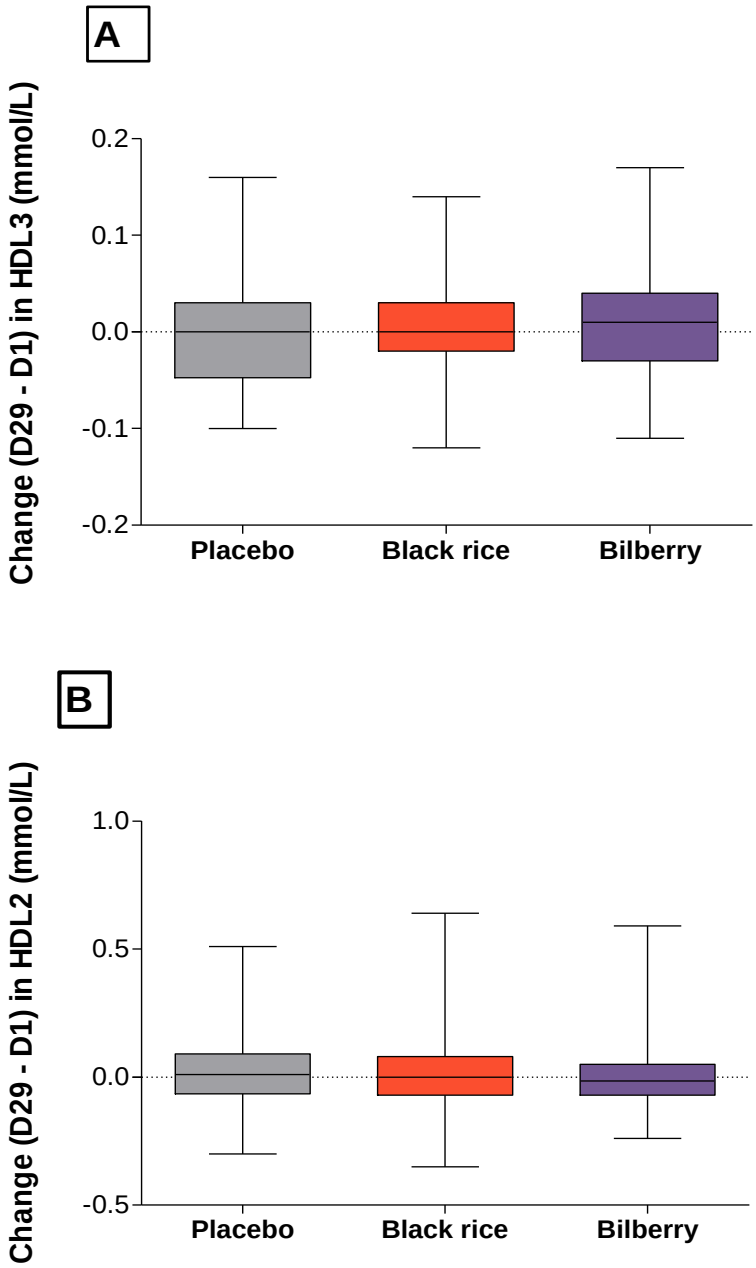


Figure 5. 12: Change in (A) HDL3 subfraction level and (B) HDL2 subfraction level in serum of humans after 28 days dietary intervention.

HDL3 and HDL2 subfractions levels in serum of all 52 hypercholesterolemic individuals were quantified at the start and end of the intervention with bilberry or black rice extract capsules that provided 320 mg/day anthocyanins. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). One measurement was conducted for each participant.

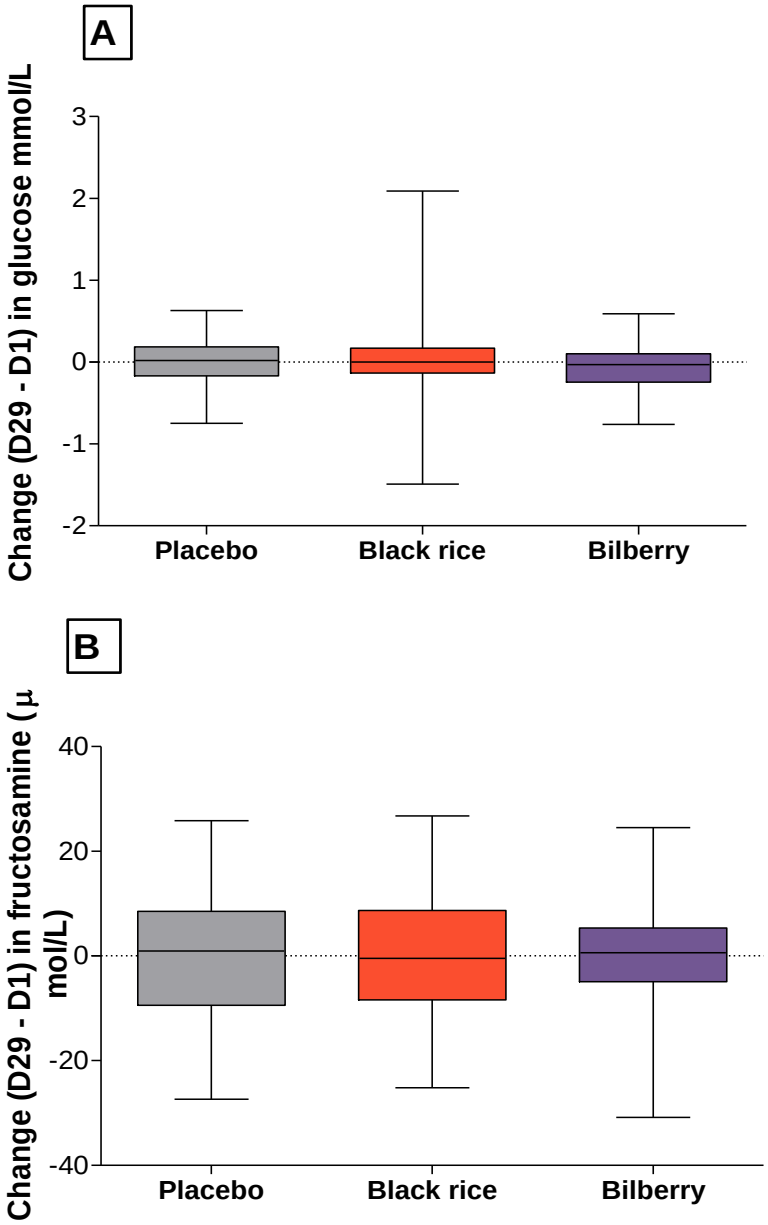


Figure 5. 13: Change in (A) glucose concentration and (B) fructosamine concentration in serum of humans after 28 days dietary intervention.

Glucose and fructosamine levels in serum of all 52 hypercholesterolemic individuals were quantified at the start and end of the intervention with bilberry or black rice extract capsules that provide 320 mg/day anthocyanins. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). One measurement was conducted for each participant.

5.5.6. Effect of the treatments on PON1 arylesterase and lactonase activities.

To investigate if the consumption of delphinidin-type or cyanidin-type of anthocyanins can affect PON1 activities; arylesterase and lactonase activities were quantified in serum of all 52 participants at the start and end of the intervention. The enzyme kinetics was measured using colourimetric assays and the enzyme units were calculated using the slop of the linear part of the enzyme reaction curve for the exact reasons that mentioned earlier in section 3.5.2.1. The linearity of the enzyme reaction was obtained at 50-fold dilution of serum for arylesterase (final concentration of serum 0.2%) and 90-fold dilution of serum for lactonase (final concentration of serum 0.1%) and lasted for at least 15 min. As shown in Fig 5.14 A and B, there was no significant effect of any of the study treatments on arylesterase or lactonase activities.

In general, lactonase activity was higher than arylesterase activity by about 2-fold. The average enzyme units of arylesterase was 3.7 (rang 0.83 to 9.36 µmol min^{-1} (ml $serum)^{-1}$, while, the average enzyme units of lactonase was 6.5 (range 2 to 11.4 µmol min^{-1} (ml $serum)^{-1}$. However, both activities remained unchanged after intervention. The very high variance between individuals is probably due to the differences in genotypes. Therefore, the effect of treatments on PON1 activities in association with genotype was investigated.

5.5.7. The interaction between anthocyanins consumption, PON1 genotype and PON1 activities.

5.5.7.1. Q192R and L55M genotype

All 52 participants were genotyped for Q192R (rs662) and for L55M (rs854560) using Sanger sequencing after amplification of the exons that contain the polymorphism. The sequence then was aligned with reference genome using Ugen software to identify the genotype of each participants.

First, Genomic DNA was extracted from whole blood samples of all participants with a mean 260:280 ratio of 1.8 (range 1.7 to 2.1) indicating that DNA samples were pure and in high quality. The efficiency of PCR reactions were checked to make sure that only the targeted exons were amplified and the primers were well-designed. Therefore, after PCR amplification reaction, samples of PCR products were separated by agarose gel electrophoresis against DNA standard ladder to check the size of PCR products.

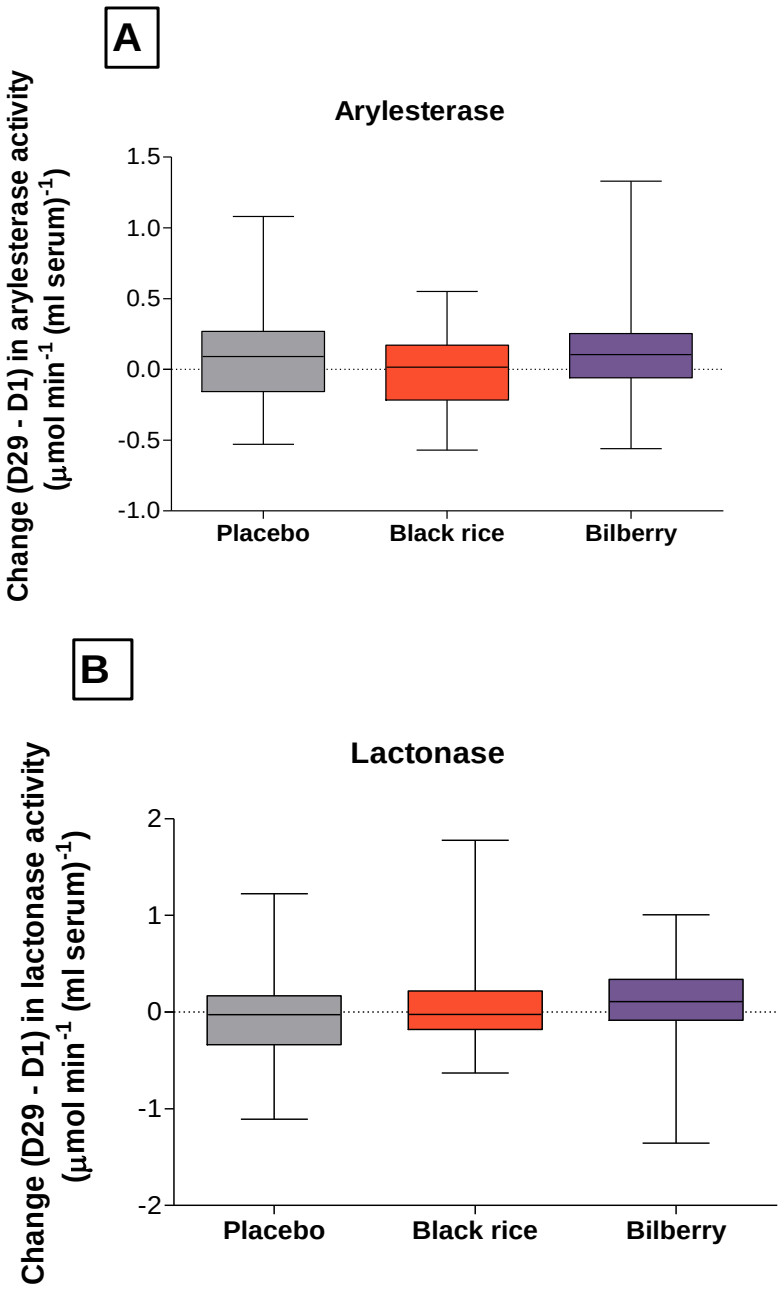


Figure 5. 14: Change in (A) arylesterase and (B) lactonase activity in serum of humans after 28 days dietary intervention.

The activities were measured in serum of all 52 hypercholesterolemic individuals at the start and end of the intervention with bilberry or black rice extract capsules that provided 320 mg/day anthocyanins using a colourimetric assay. Data are shown as means ± SD. No significant differences were detected compared with control using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). Measurements were conducted in duplicate for each participant

As shown in Fig 5.15, bands for exon 3 products (containing L55M polymorphic site) were located between 200 and 300 bp, while bands for exon 6 products (containing Q192R polymorphic site) were observed between 300 and 400 bp, indicating that the PCR products were of the correct size (240 bp for exon 3 and 333 bp for exon 6) and the PCR reaction was specific.

After the amplification of exon 3 and exon 6 for all participants, PCR products were sent for sequencing and then the genotype was determined using Ugen software. Examples of sequence of PON1 polymorphism chromatograms were shown in Fig 5.16.

Q192R and L55M genotypes and alleles distribution of subjects are reported in Table 5.4. The Q192R genotype frequencies were 42.3% QQ, 46.2% QR, and 11.5% RR, while the frequencies for L55M genotype were 42.3% LL, 48.1% LM and 9.6% MM. The frequencies of all mutations were in line with previously reported data for European population studies [367]–[373].

Table 5. 4: Genotype and allele frequency of Q192R and L55M PON1 polymorphisms in study population[1]

Polymorphism	Genotype N=52	Frequency	
		Number	%
Q192R	QQ	22	42.3
	QR	24	46.2
	RR	6	11.5
L55M	LL	22	42.3
	LM	25	48.1
	MM	5	9.6

[1] The study population was British and predominantly from the county of Norfolk. Q/R and L/M denote exchange of the amino acid at positions 192 and 55, respectively.

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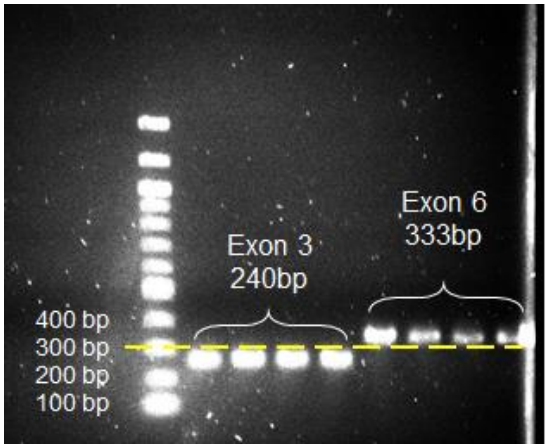


Figure 5. 15: Post electrophoresis gel image of PCR amplification products of PON1 gene.

The separation was performed in 1.5% agarose gel at 50 V for 1 hr. The gel was added to ethidium bromide solution (0.5µg/ml) and then the gel was exposed to UV light and the picture was taken with a gel imaging system.

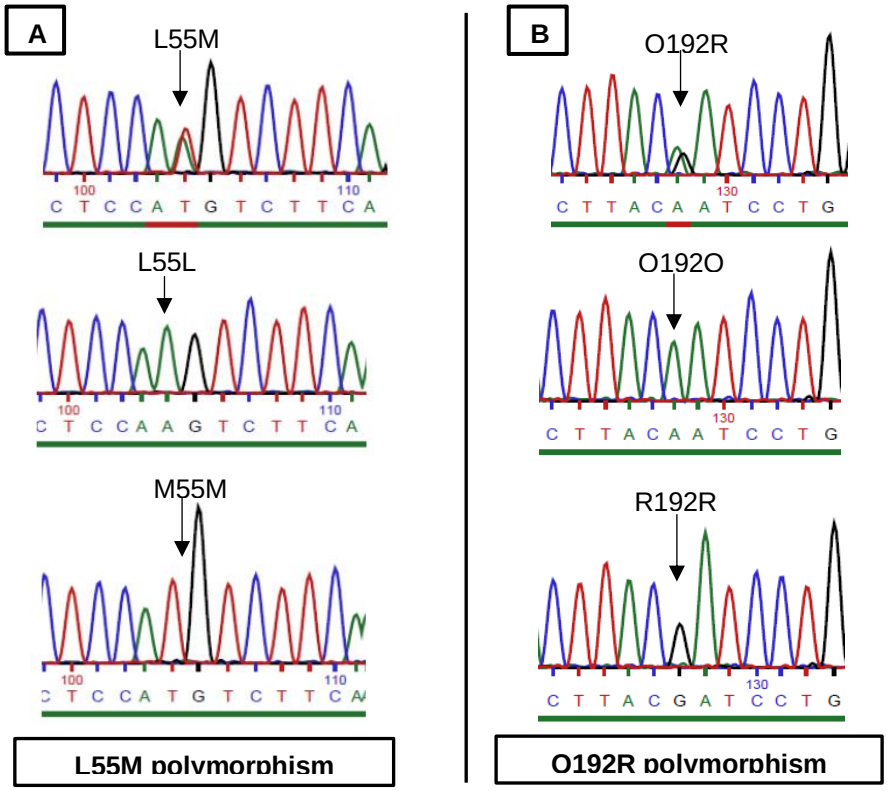


Figure 5. 16: Sequence chromatograms for (A) L55M SNP and (B) Q192R SNP

5.5.7.2. Association between PON1 genotypes and arylesterase and lactonase activity

PON1 arylesterase and lactonase activities were shown to be linked with the Q192R and L55M PON1 genotype (Table 5.5). Arylesterase was significantly higher among RR homozygotes participants followed by QR heterozygotes and QQ homozygotes ($p \leq 0.001$). People with RR genotype had arylesterase activity higher than QQ genotype by 3-fold. The average enzyme units were 6.85, 4.12 and 2.42 μmol substrate hydrolysed $(min)^{-1}$ $(ml\ serum)^{-1}$ for participants with RR, QR and QQ phenotype, respectively. Similarly, lactonase activity in participants with RR alleles was clearly higher than lactonase in participants with QQ by 1.6-fold ($p \leq 0.001$), while, the differences between QQ and QR was not significant, although lactonase activity of QR was higher than QQ.

Regarding the association between the L55M genotype and PON1 activities, arylesterase was significantly different between the three genotypes. The highest arylesterase activity was observed for participants with the LL genotype, while MM genotype was the lowest. Arylesterase in people with the LL genotype was higher than MM genotype by 2.3-fold, while the LL genotype was higher than LM by only 1.3-fold. Likewise, lactonase activity in people with LL genotype was significantly higher than people with MM. However, there was not a significant difference between people with LL or LM genotype. In addition, people with heterozygous LM genotype were significantly higher than people with the homozygous MM genotype by only 1.4-fold. The average enzyme units of lactonase were 7.2, 6.5 and 4.8 μmol substrate hydrolysed $(min)^{-1}$ $(ml\ serum)^{-1}$ for participants with LL, LM and MM genotype, respectively.

Furthermore, the association between PON1 haplotypes and PON1 activities was analysed and data are shown in Table 5.6. Of the nine possible combinations of haplotypes, eight were detected, of which two haplotype combinations (QR-MM and RR-LM) were rare with only one participant of each type. The RR-MM haplotype was not detected within the study population. The predominant haplotypes were QQ-LM, QR-LM and QR-LL which together accounted for 67% of the study population.

Table 5.6 shows the activities of PON1 as a function of PON1 haplotypes. The highest arylesterase activities was observed with the RR-LM haplotype with arylesterase activity of 9.4 μmol substrate hydrolysed $(min)^{-1}$ $(ml\ serum)^{-1}$ and lactonase activity of 11.45 μmol substrate hydrolysed $(min)^{-1}$ $(ml\ serum)^{-1}$. On the other hand, the lowest activities were observed with QQ-MM haplotype with activity of 1.4 μmol substrate

hydrolysed $(min)^{-1}$ $(ml\ serum)^{-1}$ for arylesterase and 4.01 μmol substrate hydrolysed $(min)^{-1}$ $(ml\ serum)^{-1}$ for lactonase. In addition, the analysis showed that the presence of the R allele was associated with higher arylesterase and lactonase activities; as the haplotypes of RR-LM, RR-LL, QR-LM, QR-LL and QR-MM had the highest arylesterase and lactonase activities, while, the haplotypes QQ-MM and QQ-LM had the lowest arylesterase and lactonase activity. These data indicate that the effect of the presence of L allele on enzyme activity was not as strong as allele R.

Table 5. 5: Association of PON1 Q192R and L55M polymorphism with arylesterase and lactonase activities

Polymorphism	Genotype	Arylesterase U^1/ml	Lactonase U^1/ml
Q192R	QQ	2.42 ± 0.76^{a}	5.99 ± 1.51^{ab}
	QR	4.12 ± 0.73^{b}	6.68 ± 1.16^{b}
	RR	6.85 ± 1.74^{c}	9.00 ± 2.01^{c}
L55M	L	4.42 ± 1.39^{d}	7.23 ± 1.48^{de}
	LM	3.46 ± 1.58^{e}	6.52 ± 1.41^{e}
	M	1.92 ± 1.06^{f}	4.80 ± 1.94^{f}

^{1}U is one enzyme unit= 1 μmol substrate hydrolysed $(min)^{-1}$ $(ml\ serum)^{-1}$. Values are mean ± SD. Data were statistically analysed using one-way ANOVA coupled with Newman-Keuls' multiple comparison test for arylesterase and lactonase separately. Activities with different superscript letters are significantly different (Values of $p \leq 0.05$ were considered to be significant).

Table 5. 6: Association of PON1 haplotype with arylesterase and lactonase activities

Haplotype	Frequency		Arylesterase U[1]/ml	Lactonase U[1]/ml
	Number	%		
QQ-LL	6	11.5%	3.2 ± 0.3^{a}	7.3 ± 1.3^{adf}
QQ-LM	12	23.1	2.3 ± 0.5^{b}	5.9 ± 0.7^{abce}
QQ-MM	4	7.7	1.4 ± 0.5^{c}	4.0 ± 1.5^{j}
QR-LL	11	21.2	4.2 ± 0.8^{d}	6.6 ± 1.2^{cde}
QR-LM	12	23.1	4.1 ± 0.7^{f}	6.7 ± 1.2^{b}
QR-MM	1	1.9	3.92^{j}	7.96^{h}
RR-LL	5	9.6	6.3 ± 1.4^{f}	8.5 ± 1.8^{f}
RR-LM	1	1.9	9.36^{h}	11.45^{i}
RR-MM	0	0		

[1]U is one enzyme unit= 1 μmol substrate hydrolysed $(min)^{-1}$ $(ml\ serum)^{-1}$. Values are mean ± SD. Data was statistically analysed using one-way ANOVA coupled with Newman-Keuls' multiple comparison test for arylesterase and lactonase separately. Activities with different superscript letters are significantly different (Values of $p \leq 0.05$ were considered to be significant).

5.5.7.3. Interaction between treatments and PON1 activities according to PON1 genotypes.

All participants were categorized into groups according to their Q192R and L55M genotypes. The average changes in PON1 activities after treatments were quantified for each genotypic group and analysed to investigate the interaction between treatments and PON1 activities in relation to the genotype. As shown in Table 5.7, no significant effects were observed in either arylesterase or lactonase activities after treatments whatever the genotype.

Likewise, the interaction between treatments and activities in association with Q192R and L55M haplotypes were investigated after classifying the participants into different groups according to their haplotype and quantifying the average of change in activities after treatment for each haplotype group. Table 5.8 showed that there is no interaction between treatments, PON1 activities and PON1 haplotypes.

5.5.7.4. Association between PON1 genotype and markers of CVD.

The association between PON1 genotype and biomarkers related to CVD are shown in Table 5.9. The analysis was conducted with values at baseline for each biomarker. The results show there were no associations between Q192R and L55M polymorphisms and lipid/lipoprotein profiles, HDL subfractions or glycaemic control indexes.

5.5.7.5. Interaction between treatments, PON1 genotype and HDL function biomarkers

After categorizing all participants according to their Q192R and L55M genotype; the interaction between treatments, genotype and biomarkers of HDL function was investigated. The average change in total HDL, HDL3, HDL2 and Apo A1 protein was quantified in each genotype group after consumption of bilberry extract and black rice extract powders and the effect was compared to placebo treatment. As shown in table 5.10, no significant changes were observed in total HDL, HDL3, HDL2 and Apo A1 levels within PON1 genotype after either bilberry extract or black rice extract intervention.

Table 5. 7: Effect of anthocyanins consumption on PON1 activities with association with PON1 genotype[1]

Polymorphism	Genotype	Change in arylesterase U[2]/ml			Change in lactonase U[2]/ml		
		Placebo	Black rice extract	Bilberry extract	Placebo	Black rice extract	Bilberry extract
Q192R	QQ	0.11 ± 0.25	0.00 ± 0.26	0.07 ± 0.22	-0.06 ± 0.57	0.13 ± 0.52	0.03 ± 0.32
	QR	0.15 ± 0.43	-0.05 ± 0.28	0.11 ± 0.34	0.00 ± 0.38	-0.06 ± 0.23	0.13 ± 0.50
	RR	-0.09 ± 0.29	0.08 ± 0.40	0.07 ± 0.68	-0.25 ± 0.22	-0.01 ± 0.32	0.03 ± 0.59
L55M	L	0.13 ± 0.33	0.04 ± 0.30	0.02 ± 0.41	0.03 ± 0.48	0.09 ± 0.35	-0.03 ± 0.56
	LM	0.07 ± 0.38	-0.04 ± 0.27	0.15 ± 0.28	-0.09 ± 0.41	-0.01 ± 0.43	0.16 ± 0.30
	M	0.13 ± 0.18	-0.15 ± 0.15	0.05 ± 0.09	-0.25 ± 0.46	-0.09 ± 0.24	0.10 ± 0.17

[1] The activities were measured in serum of all 52 hypercholesterolemic individuals at the start and end of the intervention. Bilberry and black rice extract capsules provide 320 mg/day anthocyanins. The participants were grouped according to their genotype. The change was calculated within each genotype group. Data are shown as means ± SD. No significant differences were detected compared with placebo using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). Baseline enzyme units of PON1 were arylesterase (0.83 to 9.36 μmol substrate hydrolysed $(min)^{-1}$ (ml serum)$^{-1}$) and lactonase (2 to 11.4 μmol substrate hydrolysed $(min)^{-1}$ (ml serum)$^{-1}$)

[2] U is one enzyme unit= 1 μmol substrate hydrolysed $(min)^{-1}$ (ml serum)$^{-1}$

Table 5. 8: Effect of anthocyanins consumption on PON1 activities with association with PON1 haplotypes[1]

Haplotype[3]	Change in arylesterase U[2]/ml			Change in lactonase U[2]/ml		
	Placebo	Black rice extract	Bilberry extract	Placebo	Black rice extract	Bilberry extract
QQ-LL	0.19 ± 0.27	0.09 ± 0.27	-0.07 ± 0.34	0.18 ± 0.83	0.43 ± 0.32	-0.13 ± 0.50
QQ-LM	0.07 ± 0.26	-0.02 ± 0.31	0.14 ± 0.16	-0.15 ± 0.41	0.07 ± 0.63	0.10 ± 0.23
QQ-MM	0.06 ± 0.15	-0.09 ± 0.14	0.08 ± 0.08	-0.23 ± 0.59	-0.08 ± 0.30	0.08 ± 0.21
QR-LL	0.18 ± 0.38	-0.05 ± 0.33	0.03 ± 0.30	0.07 ± 0.29	-0.08 ± 0.29	0.02 ± 0.62
QR-LM	0.09 ± 0.50	-0.02 ± 0.24	0.19 ± 0.38	-0.04 ± 0.46	-0.03 ± 0.18	0.22 ± 0.38
RR-LL	-0.05 ± 0.30	0.20 ± 0.30	0.12 ± 0.74	-0.24 ± 0.25	0.05 ± 0.31	-0.01 ± 0.66

[1] The activities were measured in serum of all 52 hypercholesterolemic individuals at the start and end of the intervention. Bilberry and black rice extract capsules provide 320 mg/day anthocyanins. The participants were grouped according to their haplotype. The change was calculated within each group. Data are shown as means ± SD. No significant differences detected compared with placebo using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). Baseline enzyme units of PON1 were arylesterase (0.83 to 9.36 μmol substrate hydrolysed $(min)^{-1}$ (ml serum)$^{-1}$) and lactonase (2 to 11.4 μmol substrate hydrolysed $(min)^{-1}$ (ml serum)$^{-1}$)

[2] U is one enzyme unit= 1 μmol substrate hydrolysed $(min)^{-1}$ (ml serum)$^{-1}$

[3] Haplotypes QR-MM, RR-LM and RR-MM were excluded from table because the number of particpiants within each haplotype was one or zero.

Table 5. 9: Association of PON1 Q192R genotype with markers of CVD[1]

	QQ	QR	RR	LL	LM	MM
HD (mmol/L)	1.83 ± 0.49	1.75 ± 0.38	2.16 ± 0.56	1.85 ± 0.56	1.79 ± 0.31	1.89 ± 0.72
LDL (mmol/L)	3.58 ± 0.65	4.24 ± 0.92	3.66 ± 0.72	3.81 ± 0.63	3.99 ± 0.95	3.77 ± 1.21
Cholesterol (mmol/L)	5.59 ± 0.73	6.26 ± 1.00	6.09 ± 0.64	5.89 ± 0.64	6.04 ± 1.05	5.89 ± 1.28
Triglyceride (mmol/L)	1.10 ± 0.42	1.43 ± 0.70	0.92 ± 0.32	1.19 ± 0.40	1.29 ± 0.71	1.08 ± 0.63
ApoA1 (md/dL)	223.4 ± 52.3	221.4 ± 57.4	274.4 ± 94.8	246.9 ± 84.5	210.7 ± 12.3	235.4 ± 76.7
Apo B (md/dL)	117.0 ± 14.6	139.44 ± 28.9	124.2 ± 14.9	126.0 ± 16.8	130.5 ± 27.4	126.4 ± 40.2
HDL3 (mmol/L)	0.70 ± 0.11	0.73 ± 0.10	0.74 ± 0.07	0.71 ± 0.10	0.73 ± 0.09	0.67 ± 0.12
HDL2 (mmol/L)	1.13 ± 0.42	1.02 ± 0.30	1.42 ± 0.50	1.15 ± 0.48	1.06 ± 0.24	1.22 ± 0.60
Glucose (mmol/L)	5.38 ± 0.38	5.61 ± 0.39	5.26 ± 0.32	5.43 ± 0.44	5.50 ± 0.37	5.56 ± 0.30
Fructosamine (µmol/L)	272.5 ± 30.2	260.2 ± 22.6	268.39 ± 14.5	261.8 ± 23.2	267.8 ± 23.9	279.0 ± 42.3

[1] The participants were grouped according to their genotype. Values are shown as means ± SD of the baseline measurement of participants within each group. No significant differences detected between genotypes using one-way ANOVA coupled with Newman-Keuls' multiple comparison test ($p \geq 0.0$

Table 5. 10: The interaction between anthocyanins consumption, PON1 genotype and HDL function[1].

Polymorphism	Genotype	Change in total HDL (mmol/L)			Change in HDL3 (mmol/L)		
		Placebo	Black rice extract	Bilberry extract	Placebo	Black rice extract	Bilberry extract
Q192R	QQ	0.03 ± 0.19	0.04 ± 0.24	-0.02 ± 0.11	0.00 ± 0.21	-0.01 ± 0.22	-0.08 ± 0.23
	QR	0.00 ± 0.17	0.03 ± 0.14	0.02 ± 0.15	-0.06 ± 0.27	-0.01 ± 0.23	0.03 ± 0.24
	RR	0.07 ± 0.29	0.05 ± 0.24	0.03 ± 0.32	-0.16 ± 0.24	0.11 ± 0.37	-0.08 ± 0.41
L55M	LL	0.07 ± 0.21	0.06 ± 0.16	-0.01 ± 0.20	-0.03 ± 0.22	0.11 ± 0.25	-0.05 ± 0.32
	LM	-0.01 ± 0.17	0.03 ± 0.23	0.00 ± 0.13	-0.04 ± 0.27	-0.10 ± 0.20	-0.02 ± 0.21
	MM	-0.05 ± 0.17	-0.06 ± 0.12	0.03 ± 0.09	-0.13 ± 0.20	0.06 ± 0.18	0.03 ± 0.15

[1] HDL function biomarkers were measured in serum of all 52 hypercholesterolemic individuals at the start and end of the intervention. Bilberry and black rice extract capsules provide 320 mg/day anthocyanins. The participants were grouped according to their genotype. The change was calculated within each genotype group. Data are shown as means ± SD. No significant differences detected compared with placebo using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). Base line values were as follows: Total HDL (1.05 to 3.05 mmol/L), HDL3 (0.46 to 0.95 mmol/L), HDL2 (0.59 to 2.17 mmol/L) and Apo A1 (137 to 441 mg/dL).

Table 5.10 Continued[1]

Polymorphism	Genotype	Change in HDL2 (mmol/L)			Change in Apo A1 (mg/dL)		
		Placebo	Black rice extract	Bilberry extract	Placebo	Black rice extract	Bilberry extract
Q192R	QQ	-0.80 ± 7.33	3.08 ± 8.67	1.54 ± 7.41	0.02 ± 0.16	0.04 ± 0.21	-0.02 ± 0.09
	QR	0.12 ± 7.23	-0.50 ± 6.07	3.13 ± 9.55	0.01 ± 0.12	0.02 ± 0.12	0.00 ± 0.08
	RR	-0.02 ± 6.85	2.16 ± 8.94	-1.72 ± 11.76	0.07 ± 0.25	0.06 ± 0.20	0.02 ± 0.29
L55M	LL	0.06 ± 0.16	0.05 ± 0.14	-0.01 ± 0.15	0.77 ± 8.13	2.20 ± 6.65	1.75 ± 11.39
	LM	0.01 ± 0.14	0.03 ± 0.20	-0.01 ± 0.10	-0.51 ± 5.72	0.84 ± 8.88	1.24 ± 6.53
	MM	-0.06 ± 0.16	-0.05 ± 0.12	0.02 ± 0.09	-4.55 ± 9.12	-0.15 ± 5.53	5.83 ± 7.46

[1] HDL function biomarkers were measured in serum of all 52 hypercholesterolemic individuals at the start and end of the intervention. Bilberry and black rice extract capsules provide 320 mg/day anthocyanins. The participants were grouped according to their genotype. The change was calculated within each genotype group. Data are shown as means ± SD. No significant differences detected compared with placebo using one-way ANOVA coupled with Dunnett's multiple comparison test ($p \geq 0.05$). Base line values were as follows: Total HDL (1.05 to 3.05 mmol/L), HDL3 (0.46 to 0.95 mmol/L), HDL2 (0.59 to 2.17 mmol/L) and Apo A1 (137 to 441 mg/dL).

5.6. Discussion

This chapter describes the effect of dietary supplementation (BERI study) with bilberry fruit- and black rice- derived anthocyanins on lipid/lipoproteins profile and PON1 activities as biomarkers of HDL function in humans with hypercholesterolemia. The main aim of this randomized, placebo controlled, cross-over study was to compare the differences in LDL cholesterol and other lipid/lipoproteins and markers of HDL function, after ingestion of two different major types of anthocyanins found in the diet; cyanidin-type which is found in black rice extract and delphinidin-type which is found in bilberry extract. The intention was also to determine the relationship between diet mediated changes in PON1 activity and PON1 genotype. For this purpose and according to the power calculation, 52 of hypercholesterolemic patients were recruited to consume capsules containing (i) a bilberry extract providing 320 mg anthocyanins (delphinidin-type), (ii) a black rice extract providing 320 mg anthocyanins (cyanidin-type) and (iii) a placebo control, once daily for 28 days.

The main findings in the BERI study were that (1) the intervention did not affect LDL cholesterol or other biomarkers related to vascular function (e.g. total cholesterol, HDL cholesterol, triglycerides and Apo B) and glycaemic control (glucose, fructosamine); (2) the intervention did not affect biomarkers of HDL function such as Apo A1, HDL subfractions and PON1 arylesterase and lactonase activity; (3) there was no interaction between consumption of anthocyanin, PON1 genotype, PON1 activities and markers of HDL function; (4) there was no association between PON1 genotype and biomarkers for CVD. These findings did not support the notion that anthocyanin consumption had a favourable effect on lipid/lipoproteins profiles and biomarkers of HDL function.

The primary outcome for this intervention was to investigate the effect of treatments on reduction of LDL-cholesterol which did not significantly change. These findings were inconsistent with several human intervention studies that were reported a reduction in LDL-cholesterol following a consumption of anthocyanins-rich extracts. Many of them used a whole fruit or extracts that contain several bioactives in addition to anthocyanins, while others used a proprietary product that contains isolated anthocyanins, Medox™. Zhu *et al.* (2013) and Qin *et al.* (2009) found that consumption of 320 mg/day purified anthocyanins (Medox™) for 12 to 24 weeks caused a 10% reduction in LDL cholesterol in hyperlipidaemic participants [165], [167]. They also reported a significant increase in HDL-cholesterol which contradict the findings in the BERI study [165], [167]. One possible reason for this contradiction

could be because the duration of the intervention in those two studies was 12 and 24 weeks which is 3 to 6 times longer than the current study. In a recent systematic review and meta-analysis conducted by Daneshzad *et al.* (2018), the duration of intervention was be one of the key points that create contradiction between reports in terms of the effect of anthocyanins on lipid profile [130]. In this systematic review, consumption of anthocyanins showed a significant effect on lipid profile only with trials that were conducted for longer than 12 weeks, which was not the case in our current study (BERI study). Moreover, the study design could be another possible reason that created differences in results between the previous two reports and the present study. The BERI study was a cross-over design, which is one of the main strengths in this study that minimises the effect of confounding variables and allows for assessing the actual effects of treatments, while, Qin *et al.* (2009) and Zhu *et al.* (2013) used parallel design.

On the other hand, other human intervention studies support the findings in the current study. Several human interventions reported a null effect of anthocyanins on lipid profiles when purified anthocyanins or anthocyanin-rich extracts were investigated. Thompson *et al.* (2017) reported non-significant effect on lipid profiles when 320 mg/day purified anthocyanins (Medox™) was consumed for 4 weeks [172]. In addition, Istas *et al.* (2019) found that (poly)phenol-rich aronia extract (116 mg/day) for 12 week has no effect on lipid profile [94]. Likewise, the same findings were reported when whole fruit materials were used. For Instance, Rodriguez-Mateos *et al.* (2019) found no effect of blueberry powder consumption for 4 weeks on blood lipids [307]. Moreover, Hollands *et al.* (2018) and Azzini *et al.* (2017) found that red orange juice that contained anthocyanins did not affect blood lipids compared to blond orange juice which did not contain anthocyanins after an intervention for four weeks [132], [141]. In addition, chokeberry juice providing 113 mg/day anthocyanins for 4 weeks did not affect the lipid profile [142]. In contrast, some other reports have shown that anthocyanins intervention increased lipid profile. For instance, Bergland *et al.* 2019 found that 320 mg/day anthocyanins (Medox™) for 16 weeks increased cholesterol and triglyceride in patients with mild dementia [173]. Probably, because the population was unhealthy and there was not a record whether the participants in control group are taking statin or other lipid lowering medication.

The BERI study was one of the most comprehensive human intervention that investigated the effect of anthocyanins on several biomarkers related to CVD and HDL function such as Apo A1, Apo B, HDL subfractions, ApoA1-AboB ratio, HDL-ApoA1 ratio and markers of glycaemic control in addition to the lipid profile.The

increase in ApoA1 and the decrease in ApoB was reported to be inversely associated with atherosclerosis [374], [375]. Additionally, the apoB/ apoA-I ratio was reported to better reflect the lipoprotein atherogenic balance in blood than any of the cholesterol ratios [60]. HDL subfractions were also measured. HDL comprises of several subfraction according to the density [209], [256]. HDL3 is smaller and denser and can carry more cholesterol than HDL2 subfraction [376], [377]. An increased HDL3 fraction is inversely correlated with increased CVD risk [238]. In addition, epidemiological evidence also supports an association between glycaemic control and CVD risk [378]. Similar to the lipid profile, the treatments in the current study did not affect any of these biomarkers. Likewise, Medox™ consumption and anthocyanin-containing blood orange did not affect glucose or fructosamine levels [141], [173]. Contrary, Curtis *et al.* (2019) found that the consumption of blueberry fruit (26 g/day freeze-dried for 6 months) increased Apo A1 and HDL particle density. Probably, because the duration of study was longer and they used a whole fruit that contained other bioactives which may synergistically increase the effect of anthocyanins [139].

In the current study, participants with cholesterol higher than normal were recruited. The reason was because the recent systematic review by Wallace *et al.* (2016) in which anthocyanins significantly affected LDL-cholesterol only among dyslipidaemic individuals [164]. In this systematic review, the impact of purified anthocyanins and anthocyanin-rich extracts on biomarkers of CVD risk was assessed in twelve randomized trials. Nine Of twelve intervention included LDL-cholesterol as an outcome. Of these nine trials, only four reported improvements in LDL cholesterol. These four studies were conducted in hyperlipidaemic population with a total cholesterol and LDL-cholesterol at baseline was ≥ 5.17 mmol/L and 3.8 μol/L, respectively. The other five trials which conducted in healthy individuals did not show significant effect on cholesterol or LDL-cholesterol. The authors concluded that anthocyanins significantly improved LDL-cholesterol among individuals with elevated cholesterol.

Regarding the extracts used in this intervention, the analysis of bilberry extract (*Myrti*PRO®) and black rice extract revealed that these two anthocyanin-rich extracts were suitable for the purpose of the trial. Black rice contains only dihydroxylated-B ring anthocyanins (cyanidin-type) and bilberry extract contains mainly tri-hydroxylated-B ring anthocyanins (delphinidin-type). In fact, the analysis showed that bilberry extract contains 44% delphinidin (trihydroxylated-B ring anthocyanin), while contains 24% petunidin and 8.8% malvidin. Petunidin and malvidin are methylated anthocyanins derived from delphinidin which mean that the bilberry extract represents

mainly the delphinidin types of anthocyanins (76.8%). The analysis also showed that the extracts were pure and contain mainly anthocyanins. Thereby, the effects were directly linked to anthocyanin. Additionally, the dose of 320 mg/day anthocyanins was chosen because it has previously been reported to be safe, well tolerated and have biological effects [136], [165], [167], [170], [379]. Doses up to 640 mg/day showed no adverse events [164]. However, the findings did not support the notion that anthocyanin consumption improve the lipid profiles.

In the current study, it was suggested that separating the study phases with at least 4 weeks washout is long enough to eliminate any carryover effects. This suggestion based on several human interventions that reported significant effects on cholesterol and LDL-cholesterol in crossover studies with two weeks washout period which is shorter than the washout phase used in the current study [380], [381]. Other studies reported significant effect in crossover studies with washout period of 4 weeks supporting the notion that 4 weeks is long enough for cholesterol and LDL-cholesterol to back to normal after treatments [382]. In addition, it is unlikely that the carryover effects confounded the effect of treatments as the levels of LDL-cholesterol and all measured parameters remained unchanged after all the study treatments.

Human variation (e.g. differences in microbiota or absorption and metabolism of bioactives) may be one of the factors modifying response to constituents of food [383]. Therefore, the inter-individual differences in response to treatments among the participants were checked to figure out whether there is heterogeneity across the study population in responding to treatments and whether this could help to understand the null result obtained in this study. First 88 % of study population responded similarly to treatments, supporting the notion that there is no inter-individual variation toward the treatments. Secondly, in the participants who responded differently, there was not a specific trend to explain this variation. For example, one participant reported reduced LDL (~ - 28%) with one treatment and increased LDL (~18%) with another treatment, reported a decrease in HDL from ~-2 % to ~ 17% and responded equally in the other biomarkers, indicating that the odd response is not attribute to treatment.

5.6.1. Interaction between treatments, PON1 activities and PON1 genotype.

In the current study, the effect of purified anthocyanins on two PON1 activities, arylesterase and lactonase activity was investigated. No significant effect of either black rice extract or bilberry extract was observed in either arylesterase or lactonase

activity. Few reports have investigated the effect of polyphenols on PON1 activity in humans. Only one report used purified anthocyanins and another used pomegranate juice to study their effects on arylesterase and no one investigated the effects on lactonase, the native activity of PON1. The BERI study is the first trial to report the effect of consumption of anthocyanins on PON1 lactonase activity. Zhu *et al.* (2014) reported that 320 mg/day purified anthocyanins (Medox™) for 24 weeks increased arylesterase by 17% in 122 hypercholesteraemic subjects which contradict the current findings [170]. Two reasons could explain this contradiction. First, the duration of study was 6 times longer than the BERI study. Secondly, I measured the PON1-mediated activity by using a potent inhibitor and excluded all other interfering arylesterases which was not the case in the previous report. Zhu *et al.* measured the total arylesterases without eliminating the activities of other interfering enzymes, thus, it is hard to tell whether treatment affected PON1 or other arylesterases.

It is well established that PON1 polymorphism affect the enzyme activities, stability, antiatherogenicity and sometimes the substrate's specificity [269], [270], [365], [366], [372] [260], [268], [285], [384]. Among all of PON1 SNPs, Q192R and L55M polymorphism are the most studied polymorphisms and they have previously been found to be associated with CVD, diabetes and obesity [362], [363], [373], [385], [386]. Thereby, these two polymorphisms were the main focus in my project. People with R allele has higher arylesterase activity than Q allele; and people with L allele has activity higher than M allele [387], [388]. However, some reports indicated that people with QQ possess a greater protection against LDL oxidation [266]. Thereby the genetic background related to PON1 should be considered while investigating PON1 which was the case in the BERI study. To the best of my knowledge, the BERI study is the first study to investigate the interaction between the consumption of anthocyanins, PON1 activities and PON1 genotype. In line with previous studies, there were differences between genotypes in activities in the BERI study population. It was found that RR has high arylesterase and lactonase activity than QQ.; and LL has high arylesterase and lactonase. Moreover, there was a significant association between PON1 haplotype and PON1 activities confirming the importance of categorizing the participants into different groups according to their genotype.

In addition to the polymorphisms, PON1 activities can be affected by life style and food components [389]. Therefore, the interaction between diet, PON1 activities and PON1 genotype should be further investigated. Rizzi *et al.* (2016) studied the interaction between polyphenol and PON1 variants on markers of CVD such as lipid profile using a nutrigenetic approach. They found an association with anthocyanins

intake and HDL level in interaction with some PON1 polymorphisms [273]. However, no significant interaction between treatments, PON1 activities and genotypes was observed in the current study. Probably, because studying the interaction between diet and genotype require big population size and population size might be small to detect the interaction. The power calculation was done according to LDL-cholesterol, the primary outcome, not for PON1. Therefore, not all PON1 genotypes were adequately represented in the study population and they might show a significant interaction in bigger populations. Should human interventions be undertaken in future to investigate this aspect further, the power calculation should be done according to PON1 to recruit representative study population. However, the non-significant interaction trend seen in this study doesn't support this notion as the change in activities were close to zero even in the well-represented genotypes groups such as QQ-LM, QR-LL and QR-LM.

Since PON1 binds with HDL via Apo A1, it is possible that treatments interact with HDL, HDL subfractions and Apo A1 in relation to PON1 genotype and enhance HDL function. However, there was not any significant effect of treatments on HDL function biomarkers in any of PON1 genotype groups. Possibly, other PON1 polymorphisms may interact with anthocyanins consumption and deliver preferable effects. For instance, T (-107) C polymorphism was associated with PON1 activity and HDL-cholesterol level. Therefore, more investigation still needs to be done to study the interaction of anthocyanins, more PON1 polymorphisms and markers of HDL function in big population.

5.6.2. The strengths and weaknesses of the BERI study.

There are several points of strength for this trial. This was reflected by the comprehensive analysis of biomarkers of CVD including the lipid/lipoproteins profiles and HDL function. The trial measured for the first time the effect of anthocyanin consumption on HDL subfractions and PON1 lactonase activity; and described for the first time the interaction between PON1 genotype and anthocyanin consumption in relation to biomarkers of CVD. Moreover, the trial examined the effect of purified anthocyanins so any observed effects can be directly linked to the anthocyanin. In addition, the trial included a placebo control treatment that was identical in composition and appearance to the anthocyanin treatment except for the independent variables. Control group is essential to eliminate the effects of the external factors other than the effect of treatment and provide direct comparison between anthocyanin-rich and -null food [390]. Additionally, the study was double-blinded

crossover designed. Unlike the parallel design, the crossover design allows each participants to act as his/her own control and remove between-subject variability and any other confounding factors which gives more precise estimate of treatment compared with parallel design [391]–[394]. Furthermore, the study recruited good number of participants with hypercholesterolaemia and the compared composition of the materials used in trial was identified

On the other hand, the trial was limited by the short duration due to its crossover design and therefore, further long-term studies are recommended. Another limitation was not measuring ox-LDL the main factor that initiate atherosclerosis and the ultimate target of PON1. The reason of not measuring ox-LDL was discussed in section 6.4 in this thesis.

5.7. Conclusion

In conclusion, in the undertaken human intervention trial, the consumption of 320 mg/day of bilberry extract or black rice extract derived anthocyanins for 28 days did not significantly changed the lipid/lipoprotein profile, biomarkers of HDL function, glycaemic control index or PON1 arylesterase and lactonase activities. The analysis also showed that there was no interaction between treatments, PON1 activities and PON1 genotype. The lack of effects may be due to the short duration of the intervention. Therefore, conducting interventions longer than 12 weeks should be considered in future research.

CHAPTER SIX

General discussion

6.1. Summary of main findings

The overall aims of this thesis were to (1) assess the ability of anthocyanins and their metabolites to modulate the reverse cholesterol transport (RCT) pathway and alter HDL function as mechanisms by which anthocyanins would improve lipid/lipoprotein profiles, (2) identify possible underlying mechanisms for the atheroprotective effects of anthocyanins and (3) investigate the ability of purified anthocyanins to improve lipid/lipoprotein profiles and markers of HDL function in human in a well-controlled dietary intervention study. The main findings described in this are summarized as follows:

- None of C3G, D3G or their major metabolites/mixtures at physiologically relevant concentrations significantly changed the expression of the cholesterol transporter genes or the expression of scavenger receptor genes (markers of RCT) either in cultured human macrophages or in tissues isolated from ApoE$^{-/-}$ mice fed with anthocyanin-and flavonol-expressing tomatoes.
- Neither anthocyanin parent compounds, their tested metabolites or mixtures of metabolites affected the expression of the PON1 gene (a marker of HDL function), nor did they affect PON1 arylesterase and lactonase activities, regardless of PON1 phenotype.
- Cyanidin, the aglycone form of C3G, significantly decreased the lactonase activity of PON1 but only for the RR phenotype and only at super-physiological concentrations.
- Further, the evidence that the major anthocyanin metabolites were not effective at causing significant changes in the expression of genes was extended to the entire transcriptome of human macrophages using RNA-Seq.
- Daily consumption of cyanidin- or delphinidin-type anthocyanins for 4 weeks caused no significant changes in lipid/lipoprotein profiles, markers of HDL function and PON1 activities, nor were interactions with the Q192R and L55M PON1 genotype observed.

6.2. Novelty of the presented work

The rationale for the notion that the biological activities of anthocyanins were most likely mediated by their metabolites [91] [73], [395] was presented in the general introduction (Chapter one). However, there are only a few reports describing studies of the biological effects of these metabolites [301], [302], [395]. Only two reports could be found in the scientific literature that included evidence of the effects of PCA, vanillic

acid, ferulic acid or gallic acid on markers of RCT [227], [228] and no studies concerned with the effects on PON1 gene expression and enzyme activities. In this thesis, it was investigated for the first time the possible effects of other known and potential metabolites of C3G and D3G on markers of RCT. In addition, the effect of the pure synthetic phase-2- conjugates of the major anthocyanin degradation products on markers of RCT were investigated for the first time. Moreover, this is the first *in vitro* study that has examined the effect of anthocyanins and their metabolites on PON1 gene expression and enzyme activities. Additionally, most of the previous studies examined the effects of metabolites individually and did not consider that anthocyanin metabolites are present in mixtures in vivo and they most likely interact together if they cause effects. This study is the first to look for the potential synergistic effects of anthocyanin metabolites on markers of RCT and PON1.

In the human intervention study presented in this thesis, a direct comparison between the effects of two predominant types of anthocyanin (cyanidin- and delphinidin-types) on markers of CVD was conducted for the first time. Additionally, this intervention is one of the most comprehensive trials to study the effects of consuming anthocyanins on lipid/lipoprotein profiles and to include several markers of HDL function such as HDL sub-fractions.

PON1 lactonase is the physiologically relevant activity that associated with PON1 biological functions [260]–[262]. However, paraoxonase and arylesterase were the only activities that measured in all the previous studies that investigated the effect of polyphenols on PON1. The human intervention study and the *in vitro* work presented in this thesis are the first that examined the effect of anthocyanins on PON1 lactonase activity, providing unique data concerned with another aspect of how anthocyanins might modulate HDL function.

Furthermore, it was shown in chapter three that PON1 has many polymorphisms associated with CVD risk and they often affect its enzyme activities. Therefore, it is recommended to compare between values of PON1 levels within each different genotype instead of a general comparison of the whole study population. However, the genetic background of participants has not been taken into account in any of the previous reports that investigated the effects of anthocyanins and other polyphenols on PON1 activities. This study stands out from others as it is the only one that has considered the interaction of anthocyanins with CVD risk factors in the context of PON1 polymorphisms. Thus, data from the intervention study described in this thesis is the only observational clinical trial that has investigated the interaction and the association between consumption of anthocyanins, PON1 genotype, PON1 activities

and markers of CVD. In the pre-existing literature, the only study that studied the association between consumption of anthocyanins with PON1 genotype and markers of CVD was a nutrigenomic study and not clinical trial [273].

6.3. Does the consumption of anthocyanin improve lipid/lipoprotein metabolism and what are the underlying mechanisms?

As detailed in Chapter 1, higher levels of consumption of dietary anthocyanins is associated with decreased risk of cardiovascular and other cardiometabolic diseases, and dietary interventions with anthocyanin-rich extracts and anthocyanin-rich foods has been reported to cause significant improvements in circulating lipoprotein profiles in human participants. However, it should be noted that there were significant limitations in most of these studies; typical examples include the lack of a proper control group and the use of whole fruits or whole fruit extracts which contained many other bioactives alongside the anthocyanins. Wallace *et al.* (2016) in a systematic review of intervention studies, concluded that anthocyanins may have the potential to affect markers of CVD [164]. However, the authors also recommended that more carefully controlled trials were needed to draw more concrete conclusions [164]. To investigate whether the consumption of anthocyanins does cause beneficial changes in lipid profiles and to explore the mechanisms of action, a randomised, placebo-controlled crossover trial which is one of the most rigorous, robust and comprehensive studies was conducted and accompanied with a series of *in vitro* experiments to examine the effects of anthocyanins and their metabolites on various biomarkers related to lipid/lipoprotein metabolism. In light of the previous literature and the apparent consensus view that consumption of anthocyanins favourably affects lipoprotein profiles and reduces CVD risk, it was somewhat unexpected to find no evidence in the studies reported here to support the notion that anthocyanins improve lipid/lipoprotein profiles. One possibility is that the previously reported effects were attributed to the interaction between anthocyanins and other bioactives in complex foods and crude extracts (as reported previously [42], [162], [166], [175]) and such effects are not observed when isolated anthocyanins are consumed (as reported here [131], [171], [173]). After reviewing the literature during the preparation of this thesis (summer 2019), 7 articles describing the outcomes from 5 different dietary intervention studies that examined the effect of isolated anthocyanins on lipid profiles were found, all of them describing studies that used Medox™. In only two of the studies (reported in 4 separate articles) reported significant effects on lipid profiles [136], [167], [169], [170]. Whereas, no significant effects on lipid profiles were reported

for the other three studies [131], [171], [173]. The differences between the studies for which significant effects were reported compared to those where significant effects were not observed were the duration of studies and the health status of the participants. In the two studies that reported significant effects, the duration was 12-24 weeks and the participants were hypercholesterolemic. In contrast, the duration of two of the studies not showing significant effects the durations were 3-4 weeks (in healthy participants) and for the third the duration longer (16 weeks) but the participants were cognitively impaired. The current human intervention study was conducted in hypercholesterolemic individuals, similar to the reports with significant findings. However, the duration was shorter which may explain the contradiction in findings.

In addition to the human intervention, the in vitro studies aimed to investigate the underlying mechanisms of action of anthocyanins. In chapters 2 and 3, the RCT pathway was investigated as a possible target for anthocyanins to deliver their action since it is the main way for regulating the accumulation of cholesterol in foam cells and therefore the development of atherosclerosis [214], [220], [396], [397]. In those two chapters, the influence of pure anthocyanins and their pure metabolites on the key players in RCT were investigated. To the best of my knowledge, there are only two reports that examine the effects of pure anthocyanin metabolites on the RCT pathway; these covered PCA, vanillic acid, ferulic acid and gallic acid [227], [228]. The effect of treatments on the expression of cholesterol transporter genes was measured in one study in which PCA and C3G were the only compounds tested, and PCA was reported to upregulate the expression of cholesterol transporter genes, suggesting that it is indeed the anthocyanin metabolites that interact with cells/tissues to promote RCT [228]. However, the data in this thesis does not support this notion, even though a wide range of known and predicted human metabolites of anthocyanins were studied, including the most abundant ones found in human peripheral blood after consumption of anthocyanins. As reported here in chapter two, the in vitro model was shown to be fit-for-purpose, responding strongly to the RCT-associated genes stimulator such as c-AMP and kaempferol (the positive control). These observations were consistent with gene expression data obtained from both aortic and liver tissues from mice that were fed diets supplemented with anthocyanin- and flavonol-expressing versus anthocyanin-free tomatoes, i.e. no significant effects were observed. Bearing in mind the broad range of approaches reported in this thesis, and that none of these generated any data to support the notion that anthocyanins (or their metabolites) cause favourable changes in lipid/lipoprotein profiles or biomarkers of

HDL function, it raises some doubts with regard the conclusions of the previously reported studies referred to above. Further, not only were no effects observed in a well-controlled human dietary intervention study (chapter 5), a well-controlled animal dietary intervention study (chapter 2), and a fairly comprehensive survey of the effects of anthocyanins and their human metabolites on the expression of key genes involved in the RCT pathway (chapter 2) and on PON1 expression and activity (chapter 3), no significant effects of the major human anthocyanin metabolites on the expression of any genes was observed for cultured human macrophages. Taken together, the data presented in this thesis support the notion that isolated anthocyanins and their human metabolites cannot themselves (in isolation) cause beneficial changes in lipoprotein profiles, and therefore that previously reported effects of anthocyanin-rich foods and extracts on lipoproteins are due to other components in the treatments, or combination effects with anthocyanins.

In summary, the unique data presented in this thesis provide useful, high quality data that contributes to our knowledge of the role of anthocyanins in modulating lipid/lipoprotein profiles and indeed raises several questions regarding the current ideas of their importance presented in the scientific literature

6.4. Limitations of the research

There are some limitations in the presented work in this thesis. Although the data in chapter two gave a good picture about the effect of wide range of anthocyanin metabolites at different physiologically relevant concentrations on key molecular players in the RCT process, the findings showed only the effects on gene expression with no further investigation on other regulatory elements or at the protein level. If time and the availability of commercial kits had permitted, it would have been useful to conduct cholesterol efflux capacity (CEC) assays to quantify the rate of cholesterol efflux from LDL-pre-loaded cultured macrophages [396], [398]–[400]. The CEC assay would reflect the overall effects of anthocyanins on the process including the effects on all regulatory elements and proteins involved in this pathway. However, there was a quality issue with the batch of the kit that was obtained (Sigma Cat# MAK192-1KT), and eventually the company withdrew the batch completely and a new working product did not become available in time for these assays to be completed.

There were some minor limitations in the research presented in chapter three. The working hypothesis was that anthocyanin metabolites would increase PON1 via upregulating gene expression, stimulating the enzyme activities and/or increasing enzyme secretion. Since the treatments did not show any effect on gene expression

or enzyme activities, the next step should have been studying of possible effects on the secretion of the enzyme from liver cells. However, the lack of a suitable cell model did not allow such studies to be completed. HepG2 cells did not secret detectable quantities of the enzyme, precluding its use as a model for this process. The Huh7 liver cell line which was previously reported to secret the enzyme [279], [401], [402] was not available in house and it was not feasible due to the lack of time and resources to test and optimize Huh7 cell as a model.

One of the main aims of the human intervention study that presented in chapter five was to measure the effects of anthocyanins on PON1 activities as a marker of HDL function to protect LDL from oxidation [377], [403], [404]. An increase in PON1 activities as a result of treatment would lead to a reduction in ox-LDL levels, the element that initiate the atherosclerosis [405]–[407]. However, ox-LDL level has not been measured. Although, the treatments did not show any effects on PON1 lactonase activity, the substrate that was used in the assay was synthetic and may not give a true reflection of the activity towards the physiologically relevant substrate; it was shown in chapter three that PON1 activity very much depends on the type of substrate. It is possible (although quite unlikely) that there were changes in PON1 lactonase activity towards physiological substrates (i.e. lipid peroxides) induced by the dietary intervention in humans or exposure of the enzyme to anthocyanins/metabolites, but these changes were not reflected in the enzyme activity toward TBBL. Nevertheless, future studies should include measures of ox-LDL, the true physiological substrate for PON1 in relation to HDL function. The reason that ox-LDL was not measured was that the commercially available kit (OxiSelect™ Human Oxidized LDL, Cambridge Bioscience, Cat # STA-369) has a limitation to only use newly frozen samples and not with samples that have been stored for more than 6 months. Additionally, the supplier doesn't have any data on the stability of ox-LDL and whether the differences in sample age would create variance in measurement. Since, the human intervention was a crossover design and took approximately one year to complete, it was not possible to conduct the oxLDL assays retrospectively with confidence, and so the measurement of ox-LDL was not undertaken.

6.5. Recommendations for future work

The human intervention trial in this thesis showed that the fairly substantial doses of isolated anthocyanins did not cause any significant effects on lipid profiles. This finding was actually in agreement with the outcomes reported for several human studies that used the proprietary isolated anthocyanin product Medox™ for the same

duration as the human intervention reported in this thesis [131], [171], [173]. On the other hand, other reports of human dietary intervention studies with Medox™ showed a beneficial change in lipid profiles either after consumption of Medox™ for a longer period (12-24 weeks) or with crude fruit extract. This raised important questions as to whether the isolated anthocyanins improve lipid profiles only after long-term consumption, or the anthocyanins in isolation do not cause the reported effects and instead the beneficial effects are due to other components in the crude extract treatments or as a result of interaction between anthocyanins and other components. Therefore, it would be useful to conduct additional human intervention studies to answer these questions. One is needed to compare the effect of isolated anthocyanins (such as those used here or the Medox™ product) over both shorter and longer durations, e.g. 4 weeks vs 16 weeks; This could be done via repeated sampling of participants over 16 weeks in a placebo-controlled trial (parallel or preferably crossover design). In a separate trial, it would be useful to directly compare isolated anthocyanins such as the bilberry extract used here (*Myrti*PRO®) with a crude bilberry fruit extract (i.e. containing various polyphenols and possibly other bioactive components in addition to the anthocyanins). Preferably this would be placebo controlled and of crossover design, and the duration of the intervention would be informed by the first trial investigating the effect of intervention period. This should verify if a combination of anthocyanins and the other fruit bioactives is effective whereas isolated anthocyanins are not.

The data presented in chapter two showed that treatment of LDL-loaded macrophages with C3G and D3G and with a wide range of their metabolites had no effect on the expression of key RCT genes. However, the selection of D3G metabolites was based on predictions that were based on the reported metabolites from a human study conducted with bilberry extract which contains many types of anthocyanins and it is not known for certain whether the metabolites identified in this study were derived from D3G [91]. The best way to identify D3G metabolites would be to use a stable isotope-labelled compound (normally ^{13}C-labelled) and a human study using such an approach would advance our knowledge in this area. It would then be useful to investigate the putative biological effects of any newly discovered metabolites from this human study.

With regard newly discovered metabolites, recent work in the Kroon group at QIB has shown that high concentrations of homoprotocatechuic acid (3,4 dihydroxyphenyl acetic acid) are produced and accumulate when C3G is incubated with a human faecal slurry in a colon model (Emad Shehata, Priscilla Day and Paul Kroon,

unpublished data) and future work to investigate the possible biological effects of this compound is recommended.

As discussed in chapter three, PON1 has many SNPs that associated with different chronic diseases. However, the human intervention study in chapter five only focused on Q192R and L55M SNPs, as they were associated with CVD. It is possible that other PON1 SNPs interact with consumption of anthocyanin and decrease the risk of CVD. For instance, the rs 3735590 polymorphism at the 3′ UTR of the PON1 gene which is within a miRNA binding site has been reported to be associated with ischaemic stroke and thinner carotid artery intima-media thickness (IMT) [269]. Therefore, it would be interesting to undertake a human intervention study with anthocyanins in a large study population to investigate the potential interactions between anthocyanin consumption and different PON1 SNPs after sequencing the whole gene and determining the genotype of each participant.

References

[1] K. B. Pandey and S. I. Rizvi, “Plant polyphenols as dietary antioxidants in human health and disease.,” *Oxid. Med. Cell. Longev.*, vol. 2, no. 5, pp. 270–8, 2009.

[2] V. Cheynier, “Polyphenols in foods are more complex than often thought,” *Am. J. Clin. Nutr.*, vol. 81, no. 1, pp. 223S-229S, Jan. 2005.

[3] J. A. Ross and C. M. Kasum, “D IETARY F LAVONOIDS : Bioavailability, Metabolic Effects, and Safety,” *Annu. Rev. Nutr.*, vol. 22, no. 1, pp. 19–34, Jul. 2002.

[4] L. Bravo, “Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance,” *Nutr. Rev.*, vol. 56, no. 11, pp. 317–333, Apr. 2009.

[5] A. Rodriguez-Mateos, C. Heiss, G. Borges, and A. Crozier, “Berry (Poly)phenols and Cardiovascular Health,” *J. Agric. Food Chem.*, vol. 62, no. 18, pp. 3842–3851, doi: doi:10.1021/jf403757g, May 2014.

[6] M. A. Awad, A. de Jager, L. H. W. van der Plas, and A. R. van der Krol, “Flavonoid and chlorogenic acid changes in skin of ‘Elstar’ and ‘Jonagold’ apples during development and ripening,” *Sci. Hortic. (Amsterdam).*, vol. 90, no. 1–2, pp. 69–83, Oct. 2001.

[7] I. C. Arts and P. C. Hollman, “Polyphenols and disease risk in epidemiologic studies,” *Am. J. Clin. Nutr.*, vol. 81, no. 1, pp. 317S-325S, Jan. 2005.

[8] J. B. Golding, W. B. McGlasson, A. S. Grant Wyllie, and D. N. Leach, “Fate of Apple Peel Phenolics during Cool Storage,” 2001.

[9] M. D’Archivio, C. Filesi, R. Di Benedetto, R. Gargiulo, C. Giovannini, and R. Masella, “Polyphenols, dietary sources and bioavailability.,” *Ann. Ist. Super. Sanita*, vol. 43, no. 4, pp. 348–61, 2007.

[10] C. Manach, A. Scalbert, C. Morand, C. Remesy, and L. Jimenez, “Polyphenols: food sources and bioavailability,” *Am J Clin Nutr*, vol. 79, no. 5, pp. 727–747, 2004.

[11] R. Tsao, “Chemistry and biochemistry of dietary polyphenols,” *Nutrients*, vol. 2, no. 12, pp. 1231–1246, 2010.

[12] J. G. Handique and J. B. Baruah, “Polyphenolic Compounds: an Overview,” *ChemInform*, vol. 34, no. 15, Apr. 2003.

[13] S. H. Nile and S. W. Park, “Edible berries: Bioactive components and their effect on human health,” *Nutrition*, vol. 30, no. 2, pp. 134–144, Feb. 2014.

[14] H. S. Jeong *et al.*, “Effects of black raspberry on lipid profiles and vascular endothelial function in patients with metabolic syndrome,” *Phyther. Res.*, vol. 28, no. 10, pp. 1492–1498, 2014.

[15] A. Cassidy, “Berry anthocyanin intake and cardiovascular health,” *Mol. Aspects Med.*, vol. 61, pp. 76–82, 2017.

[16] P. Chou, S. Matsui, K. Misaki, and T. Matsuda, “Isolation and Identification of Xenobiotic Aryl Hydrocarbon Receptor Ligands in Dyeing Wastewater,” *Environ. Sci. Technol.*, vol. 41, no. 2, pp. 652–657, Jan. 2007.

[17] A. Castañeda-Ovando, M. de L. Pacheco-Hernández, M. E. Páez-Hernández, J. A. Rodríguez, C. A. Galán-Vidal, and J. A. Rodriguez, “Chemical studies of anthocyanins: A review,” *Food Chem.*, vol. 113, no. January 2009, pp. 859–871, 2009.

[18] T. K. McGhie and M. C. Walton, “The bioavailability and absorption of anthocyanins: Towards a better understanding,” *Mol. Nutr. Food Res.*, vol. 51, no. 6, pp. 702–713, 2007.

[19] I. Fernandes, A. Faria, C. Calhau, V. de Freitas, and N. Mateus, “Bioavailability of anthocyanins and derivatives,” *J. Funct. Foods*, vol. 7, no. 1, pp. 54–66, 2014.

[20] J.-M. Kong, L.-S. Chia, N.-K. Goh, T.-F. Chia, and R. Brouillard, “Analysis and

biological activities of anthocyanins.," *Phytochemistry*, vol. 64, no. 5, pp. 923–33, Nov. 2003.
[21] D. Del Rio, A. Rodriguez-Mateos, J. P. E. Spencer, M. Tognolini, G. Borges, and A. Crozier, "Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases.," *Antioxid. Redox Signal.*, vol. 18, no. 14, pp. 1818–92, May 2013.
[22] S. Quideau, D. Deffieux, C. Douat-Casassus, and L. Pouységu, "Plant Polyphenols: Chemical Properties, Biological Activities, and Synthesis," *Angew. Chemie Int. Ed.*, vol. 50, no. 3, pp. 586–621, Jan. 2011.
[23] C. Qin, Y. Li, W. Niu, Y. Ding, R. Zhang, and X. Shang, "Analysis and Characterisation of Anthocyanins in Mulberry Fruit," 2010.
[24] L. Chalker-Scott, "Environmental Significance of Anthocyanins in Plant Stress Responses," *Photochem. Photobiol.*, vol. 70, no. 1, pp. 1–9, Jul. 1999.
[25] T. Tsuda, "Dietary anthocyanin-rich plants: Biochemical basis and recent progress in health benefits studies," *Mol. Nutr. Food Res.*, vol. 56, no. 1, pp. 159–170, 2012.
[26] A. Crozier, I. B. Jaganath, and M. N. Clifford, "Dietary phenolics: chemistry, bioavailability and effects on health," *Nat. Prod. Rep.*, vol. 26, no. 8, p. 1001, Aug. 2009.
[27] R. L. Prior and X. Wu, "Anthocyanins: Structural characteristics that result in unique metabolic patterns and biological activities," *Free Radic. Res.*, vol. 40, no. 10, pp. 1014–1028, 2006.
[28] I. Konczak and W. Zhang, "Anthocyanins-More Than Nature's Colours.," *J. Biomed. Biotechnol.*, vol. 2004, no. 5, pp. 239–240, 2004.
[29] A. Smeriglio, D. Monteleone, and D. Trombetta, "Health effects of Vaccinium myrtillus L.: evaluation of efficacy and technological strategies for preservation of active ingredients.," *Mini Rev. Med. Chem.*, vol. 14, no. 7, pp. 567–84, 2014.
[30] A. Smeriglio, D. Barreca, E. Bellocco, and D. Trombetta, "Chemistry, Pharmacology and Health Benefits of Anthocyanins," *Phyther. Res.*, vol. 30, no. 8, pp. 1265–1286, Aug. 2016.
[31] O. M. Andersen and K. R. Markham, *Flavonoids : chemistry, biochemistry, and applications*. CRC, Taylor & Francis, 2006.
[32] J.-M. Kong, L.-S. Chia, N.-K. Goh, T.-F. Chia, and R. Brouillard, "Analysis and biological activities of anthocyanins.," *Phytochemistry*, vol. 64, no. 5, pp. 923–33, Nov. 2003.
[33] X. Su *et al.*, "Identification and quantification of anthocyanins in transgenic purple tomato," *Food Chem.*, vol. 202, pp. 184–188, 2016.
[34] D. M. Pereira, P. Valentão, J. A. Pereira, and P. B. Andrade, "Phenolics: From chemistry to biology," *Molecules*, vol. 14, no. 6, pp. 2202–2211, 2009.
[35] M. N. Clifford, "Anthocyanins - nature, occurrence and dietary burden," *J. Sci. Food Agric.*, vol. 80, no. 7, pp. 1063–1072, May 2000.
[36] F. He *et al.*, "Anthocyanins and their variation in red wines I. Monomeric anthocyanins and their color expression.," *Molecules*, vol. 17, no. 2, pp. 1571–601, Feb. 2012.
[37] M. Horbowicz, R. Kosson, A. Grzesiuk, and H. Dębski, "Anthocyanins of Fruits and Vegetables - Their Occurrence, Analysis and Role in Human Nutrition," *Veg. Crop. Res. Bull.*, vol. 68, no. 1, pp. 5–22, 2008.
[38] J. Fang, "Bioavailability of anthocyanins," *Drug Metab Rev*, vol. 46, no. 4, pp. 508–520, 2014.
[39] A. Mauray *et al.*, "Atheroprotective effects of bilberry extracts in apo E-deficient mice," *J. Agric. Food Chem.*, vol. 57, no. 23, pp. 11106–11111, 2009.
[40] X. Wu, G. R. Beecher, J. M. Holden, D. B. Haytowitz, S. E. Gebhardt, and R. L. Prior, "Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption," *J. Agric. Food Chem.*, vol. 54, no. 11, pp. 4069–4075, 2006.

[41] C. D. Kay, P. A. Kroon, and A. Cassidy, “The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products,” *Mol. Nutr. Food Res.*, vol. 53, pp. S92–S101, doi:10.1002/mnfr.200800461, May 2009.
[42] S. Gorinstein *et al.*, “Red grapefruit positively influences serum triglyceride level in patients suffering from coronary atherosclerosis: Studies in vitro and in humans,” *J. Agric. Food Chem.*, vol. 54, no. 5, pp. 1887–1892, 2006.
[43] A. Karlsen *et al.*, “Bilberry juice modulates plasma concentration of NF-κB related inflammatory markers in subjects at increased risk of CVD,” *Eur. J. Nutr.*, vol. 49, no. 6, pp. 345–355, doi:10.1007/s00394-010-0092–0, 2010.
[44] A. Karlsen *et al.*, “Bilberry juice modulates plasma concentration of NF-kappaB related inflammatory markers in subjects at increased risk of CVD,” *Eur J Nutr*, vol. 49, no. 6, pp. 345–355, doi:10.1007/s00394-010-0092–0, 2010.
[45] A. Mauray, C. Felgines, C. Morand, A. Mazur, A. Scalbert, and D. Milenkovic, “Bilberry anthocyanin-rich extract alters expression of genes related to atherosclerosis development in aorta of apo E-deficient mice,” *Nutr. Metab. Cardiovasc. Dis.*, vol. 22, no. 1, pp. 72–80, 2012.
[46] S. R. Mallery *et al.*, “Effects of human oral mucosal tissue, saliva, and oral microflora on intraoral metabolism and bioactivation of black raspberry anthocyanins,” *Cancer Prev. Res.*, vol. 4, no. 8, pp. 1209–1221, Aug. 2011.
[47] S. Y. Wang and H. S. Lin, “Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage,” *J Agric Food Chem*, vol. 48, no. 2, pp. 140–146, 2000.
[48] Y. Wang, L. Zhao, D. Wang, Y. Huo, and B. Ji, “Anthocyanin-rich extracts from blackberry, wild blueberry, strawberry, and chokeberry: Antioxidant activity and inhibitory effect on oleic acid-induced hepatic steatosis in vitro,” *J. Sci. Food Agric.*, vol. 96, no. 7, pp. 2494–2503, 2016.
[49] C. Xie, J. Kang, J. R. Chen, S. Nagarajan, T. M. Badger, and X. Wu, “Phenolic acids are in vivo atheroprotective compounds appearing in the serum of rats after blueberry consumption,” *J. Agric. Food Chem.*, vol. 59, no. 18, pp. 10381–10387, 2011.
[50] C. Del Bo, M. Roursgaard, M. Porrini, S. Loft, P. Moller, and P. Riso, “Different effects of anthocyanins and phenolic acids from wild blueberry (Vaccinium angustifolium) on monocytes adhesion to endothelial cells in a TNF-α stimulated proinflammatory environment,” *Mol. Nutr. Food Res.*, vol. 60, no. 11, pp. 2355–2366, 2016.
[51] H. Yang, Shrown & Shan Lin, “Antioxidants activity in fruits and leaves of blackberry, raspberry and strawberry varies with culltive,” *J. Agric. Food Chem.*, vol. 48, no. 2, pp. 140–146, 2000.
[52] S. Talavera *et al.*, “Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain,” *J Agric Food Chem*, vol. 53, no. 10, pp. 3902–3908, 2005.
[53] A. K. Sandhu, Y. Huang, D. Xiao, E. Park, I. Edirisinghe, and B. Burton-Freeman, “Pharmacokinetic Characterization and Bioavailability of Strawberry Anthocyanins Relative to Meal Intake,” *J. Agric. Food Chem.*, vol. 64, no. 24, pp. 4891–4899, 2016.
[54] B. A. Cevallos-Casals and L. Cisneros-Zevallos, “Stoichiometric and kinetic studies of phenolic antioxidants from Andean purple corn and red-fleshed sweetpotato,” *J Agric Food Chem*, vol. 51, no. 11, pp. 3313–3319, 2003.
[55] M. Horbowicz, R. Kosson, A. Grzesiuk, and H. Ddbski, “Anthocyanins of Fruits and Vegetables - Their Occurrence, Analysis and Role in Human Nutrition,” vol. 68, pp. 5–22, 2008.
[56] H. Zhang, Y. Shao, J. Bao, and T. Beta, “Phenolic compounds and antioxidant properties of breeding lines between the white and black rice,” *Food Chem.*, vol. 172, pp. 630–639, 2015.
[57] G. Pereira-Caro, S. Watanabe, A. Crozier, T. Fujimura, T. Yokota, and H.

Ashihara, “Phytochemical profile of a Japanese black-purple rice,” *Food Chem.*, vol. 141, no. 3, pp. 2821–2827, 2013.
[58] W. Setyaningsih, N. Hidayah, I. E. Saputro, M. Palma, and C. García Barroso, “Profile of phenolic compounds in Indonesian rice (Oryza sativa) varieties throughout post-harvest practices,” *J. Food Compos. Anal.*, vol. 54, pp. 55–62, 2016.
[59] H. Z *et al.*, “Identification of anthocyanins isolated from black rice (Oryza sativa L.) and their degradation kinetics,” *Food Res. Int.*, vol. 50, no. 2, pp. 691–697, 2013.
[60] T. Sriseadka, S. Wongpornchai, and M. Rayanakorn, “Quantification of flavonoids in black rice by liquid chromatography- negative electrospray ionization tandem mass spectrometry,” *J. Agric. Food Chem.*, vol. 60, no. 47, pp. 11723–11732, 2012.
[61] D. Prochazkova, B. I, and W. N, “Fitoterapia Antioxidant and prooxidant properties of flavonoids,” *Fitoterapia*, vol. 82, no. 4, pp. 513–523, 2011.
[62] A. S. Hansen, P. Marckmann, L. O. Dragsted, I. L. Finné Nielsen, S. E. Nielsen, and M. Gronbæk, “Effect of red wine and red grape extract on blood lipids, haemostatic factors, and other risk factors for cardiovascular disease,” *Eur. J. Clin. Nutr.*, vol. 59, no. 3, pp. 449–455, doi:10.1038/sj.ejcn.1602107, 2005.
[63] C. Tsang *et al.*, “The influence of moderate red wine consumption on antioxidant status and indices of oxidative stress associated with CHD in healthy volunteers,” *Br. J. Nutr.*, vol. 93, no. 02, p. 233, 2007.
[64] M. Aviram *et al.*, “Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation,” *Clin. Nutr.*, vol. 23, no. 3, pp. 423–433, doi:10.1016/j.clnu.2003.10.002, 2004.
[65] M. Aviram *et al.*, “Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice,” *Am J Clin Nutr*, vol. 71, no. 5, pp. 1062–1076, 2000.
[66] J. B. Blumberg, P. E. Milbury, G. Cao, R. L. Prior, and J. Blumberg, “Bioavailability of elderberry anthocyanins Bioavailablility of elderberry anthocyanins,” *Mech. Ageing Dev.*, vol. 123, no. May 2002, pp. 997–1006, 2017.
[67] U. Mülleder, M. Murkovic, and W. Pfannhauser, “Urinary excretion of cyanidin glycosides.,” *J. Biochem. Biophys. Methods*, vol. 53, no. 1–3, pp. 61–6.
[68] S. Passamonti, U. Vrhovsek, A. Vanzo, and F. Mattivi, “The stomach as a site for anthocyanins absorption from food.,” *FEBS Lett.*, vol. 544, no. 1–3, pp. 210–3, Jun. 2003.
[69] S. Talavera, C. Felgines, O. Texier, C. Besson, J. L. Lamaison, and C. Remesy, “Anthocyanins are efficiently absorbed from the stomach in anesthetized rats,” *J Nutr*, vol. 133, no. 12, pp. 4178–4182, 2003.
[70] C. Felgines *et al.*, “Absorption and metabolism of red orange juice anthocyanins in rats.,” *Br. J. Nutr.*, vol. 95, no. 5, pp. 898–904, May 2006.
[71] C. D. Kay, G. Pereira-Caro, I. A. Ludwig, M. N. Clifford, and A. Crozier, “Anthocyanins and Flavanones Are More Bioavailable than Previously Perceived: A Review of Recent Evidence,” *Annu. Rev. Food Sci. Technol.*, vol. 8, no. 1, pp. 155–180, Feb. 2017.
[72] T. Ichiyanagi, Y. Shida, M. M. Rahman, Y. Hatano, and T. Konishi, “Bioavailability and Tissue Distribution of Anthocyanins in Bilberry (*Vaccinium myrtillus* L.) Extract in Rats,” *J. Agric. Food Chem.*, vol. 54, no. 18, pp. 6578–6587, Sep. 2006.
[73] R. M. De Ferrars *et al.*, “The pharmacokinetics of anthocyanins and their metabolites in humans,” *Br. J. Pharmacol.*, vol. 171, no. 13, pp. 3268–3282, doi:10.1111/bph.12676, 2014.

[74] C. Manach, G. Williamson, C. Morand, A. Scalbert, and and C. Rémésy, “Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies.,” *The American journal of clinical nutrition*, vol. 81, no. 1 Suppl. pp. 243S-255S, 01-Jan-2005.
[75] C. Czank *et al.*, “Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: A 13C-tracer study,” *Am. J. Clin. Nutr.*, vol. 97, no. 5, pp. 995–1003, doi:10.3945/ajcn.112.049247, 2013.
[76] C. D. Kay, “Aspects of anthocyanin absorption, metabolism and pharmacokinetics in humans,” *Nutr. Res. Rev.*, vol. 19, no. 01, p. 137, 2006.
[77] S. de Pascual-Teresa, D. A. Moreno, and C. García-Viguera, “Flavanols and anthocyanins in cardiovascular health: A review of current evidence,” *Int. J. Mol. Sci.*, vol. 11, no. 4, pp. 1679–1703, 2010.
[78] P. Vitaglione *et al.*, “Protocatechuic Acid Is the Major Human Metabolite of Cyanidin-Glucosides,” *J. Nutr.*, vol. 137, no. 9, pp. 2043–2048, 2007.
[79] C. D. Kay, G. (Joe) Mazza, and B. J. Holub, “Anthocyanins Exist in the Circulation Primarily as Metabolites in Adult Men,” *J. Nutr.*, vol. 135, no. 11, pp. 2582–2588, Nov. 2005.
[80] T. Frank *et al.*, “Pharmacokinetics of Anthocyanidin-3-Glycosides Following Consumption of Hibiscus sabdariffa L. Extract,” *J. Clin. Pharmacol.*, vol. 45, no. 2, pp. 203–210, Feb. 2005.
[81] R. Bitsch, M. Netzel, T. Frank, G. Strass, and I. Bitsch, “Bioavailability and Biokinetics of Anthocyanins From Red Grape Juice and Red Wine.,” *J. Biomed. Biotechnol.*, vol. 2004, no. 5, pp. 293–298, 2004.
[82] I. Bitsch, M. Janssen, M. Netzel, G. Strass, and T. Frank, “Bioavailability of anthocyanidin-3-glycosides following consumption of elderberry extract and blackcurrant juice.,” *Int. J. Clin. Pharmacol. Ther.*, vol. 42, no. 5, pp. 293–300, May 2004.
[83] C. D. Kay, G. Mazza, B. J. Holub, and J. Wang, “Anthocyanin metabolites in human urine and serum.,” *Br. J. Nutr.*, vol. 91, no. 6, pp. 933–42, Jun. 2004.
[84] C. Felgines *et al.*, “Strawberry Anthocyanins Are Recovered in Urine as Glucuro- and Sulfoconjugates in Humans,” *J. Nutr.*, vol. 133, no. 5, pp. 1296–1301, May 2003.
[85] I. L. F. Nielsen, L. O. Dragsted, G. Ravn-Haren, R. Freese, and S. E. Rasmussen, “Absorption and Excretion of Black Currant Anthocyanins in Humans and Watanabe Heritable Hyperlipidemic Rabbits,” *J. Agric. Food Chem.*, vol. 51, no. 9, pp. 2813–2820, Apr. 2003.
[86] G. Mazza, C. D. Kay, T. Cottrell, and B. J. Holub, “Absorption of Anthocyanins from Blueberries and Serum Antioxidant Status in Human Subjects,” *J. Agric. Food Chem.*, vol. 50, no. 26, pp. 7731–7737, Dec. 2002.
[87] A. R. Rechner *et al.*, “The metabolism of dietary polyphenols and the relevance to circulating levels of conjugated metabolites.,” *Free Radic. Res.*, vol. 36, no. 11, pp. 1229–41, Nov. 2002.
[88] X. Wu, G. Cao, and R. L. Prior, “Absorption and Metabolism of Anthocyanins in Elderly Women after Consumption of Elderberry or Blueberry,” *J. Nutr.*, vol. 132, no. 7, pp. 1865–1871, Jul. 2002.
[89] M. Murkovic, U. Mülleder, U. Adam, and W. Pfannhauser, “Detection of anthocyanins from elderberry juice in human urine,” *J. Sci. Food Agric.*, vol. 81, no. 9, pp. 934–937, Jul. 2001.
[90] M. Netzel, G. Strass, M. Janssen, I. Bitsch, and R. Bitsch, “Bioactive anthocyanins detected in human urine after ingestion of blackcurrant juice.,” *J. Environ. Pathol. Toxicol. Oncol.*, vol. 20, no. 2, pp. 89–95, 2001.
[91] D. Mueller, K. Jung, M. Winter, D. Rogoll, R. Melcher, and E. Richling, “Human intervention study to investigate the intestinal accessibility and bioavailability of anthocyanins from bilberries,” *Food Chem.*, vol. 231, no. Supplement C, pp. 275–286, doi:10.1016/J.FOODCHEM.2017.03.130, Sep. 2017.

[92] E. Azzini *et al.*, “Bioavailability of strawberry antioxidants in human subjects,” *Br. J. Nutr.*, vol. 104, no. 8, pp. 1165–1173, 2010.
[93] W. Mullen, C. A. Edwards, M. Serafini, and A. Crozier, “Bioavailability of pelargonidin-3-O-glucoside and its metabolites in humans following the ingestion of strawberries with and without cream,” *J. Agric. Food Chem.*, vol. 56, no. 3, pp. 713–719, 2008.
[94] G. Istas *et al.*, “Effects of aronia berry (poly)phenols on vascular function and gut microbiota: a double-blind randomized controlled trial in adult men,” *Am. J. Clin. Nutr.*, Jun. 2019.
[95] J. Ferreira Reis *et al.*, “Action mechanism and cardiovascular effect of anthocyanins: a systematic review of animal and human studies,” *J. Transl. Med.*, vol. 14, p. 315, 2016.
[96] A. Speciale, F. Cimino, A. Saija, R. Canali, and F. Virgili, “Bioavailability and molecular activities of anthocyanins as modulators of endothelial function,” *Genes Nutr.*, vol. 9, no. 4, p. 404, Jul. 2014.
[97] M. Quinones, M. Miguel, and A. Aleixandre, “Beneficial effects of polyphenols on cardiovascular disease,” *Pharmacol. Res.*, vol. 68, no. 1, pp. 125–131, Feb. 2013.
[98] C. D. Mathers and D. Loncar, “Projections of Global Mortality and Burden of Disease from 2002 to 2030,” *PLoS Med.*, vol. 3, no. 11, p. e442, Nov. 2006.
[99] T. C. Wallace, “Anthocyanins in Cardiovascular Disease 1,” *Adv. Nutr*, vol. 2, pp. 1–7, 2011.
[100] British Heart Foundation, “Heart statistics - Heart and Circulatory Diseases in the UK - BHF,” *ey statistics and information on heart and circulatory diseases for the UK*, 2019. [Online]. Available: https://www.bhf.org.uk/what-we-do/our-research/heart-statistics. [Accessed: 02-Aug-2019].
[101] D. Xi, J. Zhao, W. Lai, and Z. Guo, “Systematic analysis of the molecular mechanism underlying atherosclerosis using a text mining approach,” *Hum. Genomics*, vol. 10, no. 1, p. 14, 2016.
[102] P. P. Toth, “Subclinical atherosclerosis: What it is, what it means and what we can do about it,” *Int. J. Clin. Pract.*, vol. 62, no. 8, pp. 1246–1254, 2008.
[103] E. Falk, “Pathogenesis of Atherosclerosis,” *J. Am. Coll. Cardiol.*, vol. 47, no. 8, pp. C7–C12, Apr. 2006.
[104] J. P. Strong *et al.*, “Prevalence and Extent of Atherosclerosis in Adolescents and Young Adults<SUBTITLE>Implications for Prevention From the Pathobiological Determinants of Atherosclerosis in Youth Study</SUBTITLE>,” *JAMA*, vol. 281, no. 8, p. 727, Feb. 1999.
[105] M. Naghavi *et al.*, “From Vulnerable Plaque to Vulnerable Patient,” *Circulation*, vol. 108, no. 15, pp. 1772–1778, Oct. 2003.
[106] M. Naghavi *et al.*, “From Vulnerable Plaque to Vulnerable Patient,” *Circulation*, vol. 108, no. 14, pp. 1664–1672, Oct. 2003.
[107] J. A. Ambrose and M. Singh, “Pathophysiology of coronary artery disease leading to acute coronary syndromes,” *F1000Prime Rep.*, vol. 7, p. 08, 2015.
[108] L. G. Spagnoli *et al.*, “Extracranial Thrombotically Active Carotid Plaque as a Risk Factor for Ischemic Stroke,” *JAMA*, vol. 292, no. 15, p. 1845, Oct. 2004.
[109] C. K. Glass and J. L. Witztum, “Atherosclerosis. the road ahead.,” *Cell*, vol. 104, no. 4, pp. 503–16, Feb. 2001.
[110] R. Virmani *et al.*, “Atherosclerotic Plaque Progression and Vulnerability to Rupture,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 25, no. 10, pp. 2054–2061, Oct. 2005.
[111] J. L. Orford, A. P. Selwyn, P. Ganz, J. J. Popma, and C. Rogers, “The comparative pathobiology of atherosclerosis and restenosis.,” *Am. J. Cardiol.*, vol. 86, no. 4B, pp. 6H-11H, Aug. 2000.
[112] P. Libby and M. Aikawa, “Stabilization of atherosclerotic plaques: New mechanisms and clinical targets,” *Nat. Med.*, vol. 8, no. 11, pp. 1257–1262,

Nov. 2002.
[113] D. Wolf, P. Stachon, C. Bode, and A. Zirlik, “Inflammatory mechanisms in atherosclerosis,” *Hamostaseologie*, vol. 34, no. 01, pp. 63–71, Dec. 2014.
[114] S. E. Nissen *et al.*, “Effect of Recombinant ApoA-I Milano on Coronary Atherosclerosis in Patients With Acute Coronary Syndromes,” *JAMA*, vol. 290, no. 17, p. 2292, Nov. 2003.
[115] G. F. Lewis and D. J. Rader, “New Insights Into the Regulation of HDL Metabolism and Reverse Cholesterol Transport,” *Circ. Res.*, vol. 96, no. 12, pp. 1221–1232, Jun. 2005.
[116] A. Rodriguez-Mateos, C. Heiss, G. Borges, and A. Crozier, “Berry (Poly)phenols and Cardiovascular Health,” *J. Agric. Food Chem.*, vol. 62, no. 18, pp. 3842–3851, May 2014.
[117] A. Cassidy, M. Bertoia, S. Chiuve, A. Flint, J. Forman, and E. B. Rimm, “Habitual intake of anthocyanins and flavanones and risk of cardiovascular disease in men,” *Am. J. Clin. Nutr.*, vol. 104, no. 3, pp. 587–594, doi:10.3945/ajcn.116.133132, Sep. 2016.
[118] A. Cassidy, K. J. Mukamal, L. Liu, M. Franz, A. H. Eliassen, and E. B. Rimm, “High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women,” *Circulation*, vol. 127, no. 2, pp. 188–196, 2013.
[119] A. Cassidy *et al.*, “Habitual intake of flavonoid subclasses and incident hypertension in adults,” *Am. J. Clin. Nutr.*, vol. 93, no. 2, pp. 338–347, 2011.
[120] P. J. Mink *et al.*, “Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women,” *Am. J. Clin. Nutr.*, vol. 85, no. 3, pp. 895–909, Mar. 2007.
[121] M. L. McCullough, J. J. Peterson, R. Patel, P. F. Jacques, R. Shah, and J. T. Dwyer, “Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults,” *Am. J. Clin. Nutr.*, vol. 95, no. 2, pp. 454–464, Feb. 2012.
[122] A. Jennings *et al.*, “Higher anthocyanin intake is associated with lower arterial stiffness and central blood pressure in women,” *Am. J. Clin. Nutr.*, vol. 96, no. 4, pp. 781–788, Oct. 2012.
[123] A. Jennings, A. A. Welch, T. Spector, A. Macgregor, and A. Cassidy, “Intakes of Anthocyanins and Flavones Are Associated with Biomarkers of Insulin Resistance and Inflammation in Women,” *J. Nutr.*, vol. 144, no. 2, pp. 202–208, Feb. 2014.
[124] J. Mursu, S. Voutilainen, T. Nurmi, T.-P. Tuomainen, S. Kurl, and J. T. Salonen, “Flavonoid intake and the risk of ischaemic stroke and CVD mortality in middle-aged Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study,” *Br. J. Nutr.*, vol. 100, no. 4, pp. 890–895, Oct. 2008.
[125] H. D. Sesso, J. M. Gaziano, S. Liu, and J. E. Buring, “Flavonoid intake and the risk of cardiovascular disease in women,” *Am. J. Clin. Nutr.*, vol. 77, no. 6, pp. 1400–1408, Jun. 2003.
[126] A. Cassidy *et al.*, “Dietary flavonoids and risk of stroke in women.,” *Stroke*, vol. 43, no. 4, pp. 946–51, Apr. 2012.
[127] K. Shah and P. Shah, “Effect of Anthocyanin Supplementations on Lipid Profile and Inflammatory Markers: A Systematic Review and Meta-Analysis of Randomized Controlled Trials,” 2018.
[128] Â. Luís, F. Domingues, and L. Pereira, “Association between berries intake and cardiovascular diseases risk factors: a systematic review with meta-analysis and trial sequential analysis of randomized controlled trials,” *Food Funct.*, vol. 9, no. 2, pp. 740–757, Feb. 2018.
[129] J. Zhan, Y.-J. Liu, L.-B. Cai, F.-R. Xu, T. Xie, and Q.-Q. He, “Fruit and vegetable consumption and risk of cardiovascular disease: A meta-analysis of prospective cohort studies,” *Crit. Rev. Food Sci. Nutr.*, vol. 57, no. 8, pp. 1650–1663, May 2017.

[130] E. Daneshzad, S. Shab-Bidar, Z. Mohammadpour, and K. Djafarian, “Effect of anthocyanin supplementation on cardio-metabolic biomarkers: A systematic review and meta-analysis of randomized controlled trials,” *Clin. Nutr.*, Jul. 2018.
[131] A. Rodriguez-Mateos *et al.*, “Circulating Anthocyanin Metabolites Mediate Vascular Benefits of Blueberries: Insights From Randomized Controlled Trials, Metabolomics, and Nutrigenomics,” *Journals Gerontol. Ser. A*, vol. 74, no. 7, pp. 967–976, Jun. 2019.
[132] E. Azzini *et al.*, “Effect of Red Orange Juice Consumption on Body Composition and Nutritional Status in Overweight/Obese Female: A Pilot Study,” *Oxid. Med. Cell. Longev.*, vol. 2017, p. 1672567, 2017.
[133] M. Aviram and L. Dornfeld, “Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure.,” *Atherosclerosis*, vol. 158, no. 1, pp. 195–8, Sep. 2001.
[134] M. D. Sumner *et al.*, “Effects of Pomegranate Juice Consumption on Myocardial Perfusion in Patients With Coronary Heart Disease,” *Am. J. Cardiol.*, vol. 96, no. 6, pp. 810–814, Sep. 2005.
[135] M. F.-F. Chong, R. Macdonald, and J. A. Lovegrove, “Fruit polyphenols and CVD risk: a review of human intervention studies,” *Br. J. Nutr.*, vol. 104, no. S3, pp. S28–S39, Oct. 2010.
[136] Y. Zhu *et al.*, “Purified anthocyanin supplementation improves endothelial function via NO-cGMP activation in hypercholesterolemic individuals,” *Clin. Chem.*, vol. 57, no. 11, pp. 1524–1533, 2011.
[137] A. J. Stull, K. C. Cash, W. D. Johnson, C. M. Champagne, and W. T. Cefalu, “Bioactives in Blueberries Improve Insulin Sensitivity in Obese, Insulin-Resistant Men and Women,” *J. Nutr.*, vol. 140, no. 10, pp. 1764–1768, Oct. 2010.
[138] I. Erlund *et al.*, “Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol,” *Am J Clin Nutr*, vol. 87, no. 2, pp. 323–331, 2008.
[139] P. J. Curtis *et al.*, “Blueberries improve biomarkers of cardiometabolic function in participants with metabolic syndrome—results from a 6-month, double-blind, randomized controlled trial,” *Am. J. Clin. Nutr.*, vol. 109, no. 6, pp. 1535–1545, Jun. 2019.
[140] M. Habanova *et al.*, “Intake of bilberries (Vaccinium myrtillus L.) reduced risk factors for cardiovascular disease by inducing favorable changes in lipoprotein profiles,” *Nutr. Res.*, vol. 36, no. 12, pp. 1415–1422, 2016.
[141] W. J. Hollands, C. N. Armah, J. F. Doleman, N. Perez-Moral, M. S. Winterbone, and P. A. Kroon, “4-Week consumption of anthocyanin-rich blood orange juice does not affect LDL-cholesterol or other biomarkers of CVD risk and glycaemia compared with standard orange juice: A randomised controlled trial,” *Br. J. Nutr.*, vol. 119, no. 4, pp. 415–421, 2018.
[142] B. Pokimica *et al.*, “Chokeberry Juice Containing Polyphenols Does Not Affect Cholesterol or Blood Pressure but Modifies the Composition of Plasma Phospholipids Fatty Acids in Individuals at Cardiovascular Risk,” *Nutrients*, vol. 11, no. 4, p. 850, Apr. 2019.
[143] A. Rodriguez-Mateos *et al.*, “Cranberry (poly)phenol metabolites correlate with improvements in vascular function: A double-blind, randomized, controlled, dose-response, crossover study,” *Mol. Nutr. Food Res.*, vol. 60, no. 10, pp. 2130–2140, Oct. 2016.
[144] L. Xie *et al.*, “Aronia berry polyphenol consumption reduces plasma total and low-density lipoprotein cholesterol in former smokers without lowering biomarkers of inflammation and oxidative stress: a randomized controlled trial,” *Nutr. Res.*, vol. 37, pp. 67–77, 2017.
[145] A. Lerman and A. M. Zeiher, “Endothelial Function,” *Circulation*, vol. 111, no. 3, pp. 363–368, Jan. 2005.

[146] R. Ross, “The pathogenesis of atherosclerosis: a perspective for the 1990s,” *Nature*, vol. 362, no. 6423, pp. 801–809, Apr. 1993.
[147] D. S. Celermajer, “Endothelial dysfunction: does it matter? Is it reversible?,” *J. Am. Coll. Cardiol.*, vol. 30, no. 2, pp. 325–33, Aug. 1997.
[148] S. Laurent *et al.*, “Expert consensus document on arterial stiffness: methodological issues and clinical applications,” *Eur. Heart J.*, vol. 27, no. 21, pp. 2588–2605, Sep. 2006.
[149] J. Yeboah *et al.*, “Predictive Value of Brachial Flow-Mediated Dilation for Incident Cardiovascular Events in a Population-Based Study,” *Circulation*, vol. 120, no. 6, pp. 502–509, Aug. 2009.
[150] L. S. McAnulty *et al.*, “Six weeks daily ingestion of whole blueberry powder increases natural killer cell counts and reduces arterial stiffness in sedentary males and females,” *Nutr. Res.*, vol. 34, no. 7, pp. 577–584, Jul. 2014.
[151] S. A. Johnson *et al.*, “Daily Blueberry Consumption Improves Blood Pressure and Arterial Stiffness in Postmenopausal Women with Pre- and Stage 1-Hypertension: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial,” *J. Acad. Nutr. Diet.*, vol. 115, no. 3, pp. 369–377, Mar. 2015.
[152] M. M. Dohadwala *et al.*, “Effects of cranberry juice consumption on vascular function in patients with coronary artery disease,” *Am. J. Clin. Nutr.*, vol. 93, no. 5, pp. 934–940, May 2011.
[153] Y. Jin, D. Alimbetov, T. George, M. H. Gordon, and J. A. Lovegrove, “A randomised trial to investigate the effects of acute consumption of a blackcurrant juice drink on markers of vascular reactivity and bioavailability of anthocyanins in human subjects,” *Eur. J. Clin. Nutr.*, vol. 65, no. 7, pp. 849–856, Jul. 2011.
[154] P. Riso *et al.*, “Effect of a wild blueberry (Vaccinium angustifolium) drink intervention on markers of oxidative stress, inflammation and endothelial function in humans with cardiovascular risk factors,” *Eur. J. Nutr.*, vol. 52, no. 3, pp. 949–961, Apr. 2013.
[155] C. Del Bo′ *et al.*, “A single portion of blueberry (Vaccinium corymbosum L) improves protection against DNA damage but not vascular function in healthy male volunteers,” *Nutr. Res.*, vol. 33, no. 3, pp. 220–227, Mar. 2013.
[156] C. Cavelier, I. Lorenzi, L. Rohrer, and A. von Eckardstein, “Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1,” *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, vol. 1761, no. 7, pp. 655–666, 2006.
[157] C. L. Millar, Q. Duclos, and C. N. Blesso, “Effects of Dietary Flavonoids on Reverse Cholesterol Transport, HDL Metabolism, and HDL Function,” *Adv. Nutr. An Int. Rev. J.*, vol. 8, no. 2, pp. 226–239, 2017.
[158] B. Knight, “ATP-binding cassette transporter A1: regulation of cholesterol efflux.,” *Biochem. Soc. Trans.*, vol. 32, no. Pt 1, pp. 124–7, 2004.
[159] D. J. McNamara, “Dietary cholesterol and atherosclerosis,” *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, vol. 1529, no. 1–3, pp. 310–320, Dec. 2000.
[160] S. Soumian, C. Albrecht, A. H. Davies, and R. G. J. Gibbs, “ABCA1 and atherosclerosis,” *Vasc. Med.*, vol. 10, no. 2, pp. 109–119, 2005.
[161] D. Ivanova, O. Tasinov, and Y. Kiselova-Kaneva, “Improved lipid profile and increased serum antioxidant capacity in healthy volunteers after Sambucus ebulus L. fruit infusion consumption,” *Int. J. Food Sci. Nutr.*, vol. 65, no. 6, pp. 740–744, 2014.
[162] S. Kianbakht, B. Abasi, and F. Hashem Dabaghian, “Improved lipid profile in hyperlipidemic patients taking vaccinium arctostaphylos fruit hydroalcoholic extract: A randomized double-blind placebo-controlled clinical trial,” *Phyther. Res.*, vol. 28, no. 3, pp. 432–436, doi:10.1002/ptr.5011, 2014.
[163] J. M. Alvarez-Suarez *et al.*, “One-month strawberry-rich anthocyanin supplementation ameliorates cardiovascular risk, oxidative stress markers and platelet activation in humans,” *J. Nutr. Biochem.*, vol. 25, no. 3, pp. 289–294,

2014.
[164] T. C. Wallace, M. Slavin, and C. L. Frankenfeld, “Systematic review of anthocyanins and markers of cardiovascular dise,” *Nutrients*, vol. 8, no. 1, pp. 32, doi:10.3390/nu8010032, 2016.
[165] Y. Qin *et al.*, “Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects,” *Am. J. Clin. Nutr.*, vol. 90, no. 3, pp. 485–492, doi:10.3945/ajcn.2009.27814, 2009.
[166] R. Soltani, M. Hakimi, S. Asgary, S. M. Ghanadian, M. Keshvari, and N. Sarrafzadegan, “Evaluation of the effects of Vaccinium arctostaphylos L. Fruit extract on serum lipids and hs-CRP levels and oxidative stress in adult patients with hyperlipidemia: A randomized, double-blind, placebo-controlled clinical trial,” *Evidence-based Complement. Altern. Med.*, vol. 2014, p. 217451, 2014.
[167] Y. Zhu *et al.*, “Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: A randomized controlled trial,” *Nutr. Metab. Cardiovasc. Dis.*, vol. 23, no. 9, pp. 843–849, Sep. 2013.
[168] C. M. Gurrola-Díaz, P. M. García-López, S. Sánchez-Enríquez, R. Troyo-Sanromán, I. Andrade-González, and J. F. Gómez-Leyva, “Effects of Hibiscus sabdariffa extract powder and preventive treatment (diet) on the lipid profiles of patients with metabolic syndrome (MeSy),” *Phytomedicine*, vol. 17, no. 7, pp. 500–505, doi:10.1016/j.phymed.2009.10.014, Jun. 2010.
[169] Y. Qin *et al.*, “Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects,” *Am. J. Clin. Nutr.*, vol. 90, no. 3, pp. 485–492, doi:10.3945/ajcn.2009.27814, Sep. 2009.
[170] Y. Zhu *et al.*, “Anthocyanin supplementation improves HDL-Associated paraoxonase 1 activity and enhances cholesterol efflux capacity in subjects with hypercholesterolemia,” *J. Clin. Endocrinol. Metab.*, vol. 99, no. 2, pp. 561–569, doi:10.1210/jc.2013-2845, Feb. 2014.
[171] A. Karlsen *et al.*, “Anthocyanins Inhibit Nuclear Factor-κB Activation in Monocytes and Reduce Plasma Concentrations of Pro-Inflammatory Mediators in Healthy Adults,” *J. Nutr.*, vol. 137, no. 8, pp. 1951–1954, Aug. 2007.
[172] K. Thompson, H. Hosking, W. Pederick, I. Singh, and A. B. Santhakumar, “The effect of anthocyanin supplementation in modulating platelet function in sedentary population: a randomised, double-blind, placebo-controlled, cross-over trial,” *Br. J. Nutr.*, vol. 118, no. 05, pp. 368–374, Sep. 2017.
[173] A. K. Bergland *et al.*, “Effects of Anthocyanin Supplementation on Serum Lipids, Glucose, Markers of Inflammation and Cognition in Adults With Increased Risk of Dementia – A Pilot Study,” *Front. Genet.*, vol. 10, p. 536, Jun. 2019.
[174] P. Duchnowicz, A. Nowicka, M. Koter-Michalak, and M. Broncel, “In vivo influence of extract from Aronia melanocarpa on the erythrocyte membranes in patients with hypercholesterolemia,” *Med. Sci. Monit.*, vol. 18, no. 9, pp. CR569–CR574, 2012.
[175] Y. S. Park *et al.*, “Characteristics of blond and red Star Ruby Jaffa grapefruits (Citrus paradise): results of the studies in vitro, in vivo and on patients suffering from atherosclerosis.,” *ActaHortic*, vol. 841, pp. 137–144, 2009.
[176] R. Poreba *et al.*, “Drinking of chokeberry juice from the ecological farm Dzieciolowo and distensibility of brachial artery in men with mild hypercholesterolemia.,” *Ann. Agric. Environ. Med.*, vol. 16, no. 2, pp. 305–8, 2009.
[177] J. Sikora, M. Broncel, M. Markowicz, M. Chałubiński, K. Wojdan, and E. Mikiciuk-Olasik, “Short-term supplementation with Aronia melanocarpa extract improves platelet aggregation, clotting, and fibrinolysis in patients with metabolic syndrome,” *Eur. J. Nutr.*, vol. 51, no. 5, pp. 549–556, 2012.

[178] M. Broncel, M. Kozirog, P. Duchnowicz, M. Koter-Michalak, J. Sikora, and J. Chojnowska-Jezierska, “Aronia melanocarpa extract reduces blood pressure, serum endothelin, lipid, and oxidative stress marker levels in patients with metabolic syndrome.,” *Med. Sci. Monit.*, vol. 16, no. 1, pp. CR28-34, 2010.
[179] A. Basu, M. Wilkinson, K. Penugonda, B. Simmons, N. M. Betts, and T. J. Lyons, “Freeze-dried strawberry powder improves lipid profile and lipid peroxidation in women with metabolic syndrome: Baseline and post intervention effects,” *Nutr. J.*, vol. 8, no. 1, p. 43, 2009.
[180] T. L. Zern *et al.*, “Grape polyphenols exert a cardioprotective effect in pre- and postmenopausal women by lowering plasma lipids and reducing oxidative stress.,” *J. Nutr.*, vol. 135, no. 8, pp. 1911–7, 2005.
[181] G. Ruel, S. Pomerleau, P. Couture, S. Lemieux, B. Lamarche, and C. Couillard, “Favourable impact of low-calorie cranberry juice consumption on plasma HDL-cholesterol concentrations in men,” *Br. J. Nutr.*, vol. 96, no. 02, p. 357, 2007.
[182] I. Erlund *et al.*, “Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol,” *Am. J. Clin. Nutr.*, vol. 87, no. 2, pp. 323–331, Feb. 2008.
[183] S. J. Zunino *et al.*, “Effects of dietary strawberry powder on blood lipids and inflammatory markers in obese human subjects,” *Br. J. Nutr.*, vol. 108, no. 5, pp. 900–909, 2012.
[184] F. H. Rached, M. J. Chapman, and A. Kontush, “HDL particle subpopulations: Focus on biological function,” *BioFactors*, vol. 41, no. 2, pp. 67–77, Mar. 2015.
[185] T. M. Forte, A. V Nichols, R. M. Krauss, and R. A. Norum, “Familial apolipoprotein AI and apolipoprotein CIII deficiency. Subclass distribution, composition, and morphology of lipoproteins in a disorder associated with premature atherosclerosis.,” *J. Clin. Invest.*, vol. 74, no. 5, pp. 1601, doi:10.1172/JCI111576, 1984.
[186] P. W. Wilson, R. D. Abbott, and W. P. Castelli, “High density lipoprotein cholesterol and mortality. The Framingham Heart Study.,” *Arterioscler. An Off. J. Am. Hear. Assoc. Inc.*, vol. 8, no. 6, pp. 737–741, doi: 10.1161/01.ATV.8.6.737, Nov. 1988.
[187] P. K. Shah and J. Amin, “Low high density lipoprotein level is associated with increased restenosis rate after coronary angioplasty.,” *Circulation*, vol. 85, no. 4, pp. 1279–85, doi:10.1161/01.cir.85.4.1279, Apr. 1992.
[188] D. J. Gordon *et al.*, “High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies.,” *Circulation*, vol. 79, no. 1, pp. 8–15, doi:10.1161/01.cir.79.1.8, Jan. 1989.
[189] D. Sviridov, N. Mukhamedova, A. T. Remaley, J. Chin-Dusting, and P. Nestel, “Antiatherogenic functionality of high density lipoprotein: how much versus how good.,” *J. Atheroscler. Thromb.*, vol. 15, no. 2, pp. 52–62, Apr. 2008.
[190] P. Roma *et al.*, “In vivo metabolism of a mutant form of apolipoprotein A-I, apo A-IMilano, associated with familial hypoalphalipoproteinemia.,” *J. Clin. Invest.*, vol. 91, no. 4, pp. 1445–1452, Apr. 1993.
[191] R. Frikke-Schmidt *et al.*, “Association of Loss-of-Function Mutations in the <emph type="ital">ABCA1</emph> Gene With High-Density Lipoprotein Cholesterol Levels and Risk of Ischemic Heart Disease,” *JAMA*, vol. 299, no. 21, p. 2524, Jun. 2008.
[192] P. J. Barter *et al.*, “Effects of Torcetrapib in Patients at High Risk for Coronary Events,” *N. Engl. J. Med.*, vol. 357, no. 21, pp. 2109–2122, Nov. 2007.
[193] Á. Hernáez *et al.*, “Mediterranean Diet Improves High-Density Lipoprotein Function in High-Cardiovascular-Risk Individuals: A Randomized Controlled Trial,” *Circulation*, vol. 135, no. 7, pp. 633–643, 2017.
[194] M. J. Haas and A. D. Mooradian, “Regulation of high-density lipoprotein by inflammatory cytokines: establishing links between immune dysfunction and cardiovascular disease,” *Diabetes. Metab. Res. Rev.*, vol. 26, no. 2, pp. 90–99,

doi:10.1002/dmrr.1057, Feb. 2010.
[195] L. M. Raffield *et al.*, “Impact of HDL genetic risk scores on coronary artery calcified plaque and mortality in individuals with type 2 diabetes from the Diabetes Heart Study,” *Cardiovasc. Diabetol.*, vol. 12, no. 1, pp. 95–105, doi:10.1186/1475-2840-12–95, Jun. 2013.
[196] P. K. Shah, “Jekyll and Hyde of HDL: a lipoprotein with a split personality,” *Eur. Heart J.*, vol. 34, no. 46, pp. 3531–3534, doi:10.1093/eurheartj/eht382, Dec. 2013.
[197] R. Movva and D. J. Rader, “Laboratory assessment of HDL heterogeneity and function,” *Clin. Chem.*, vol. 54, no. 5, pp. 788–800, 2008.
[198] A. R. Tall, L. Yvan-Charvet, N. Terasaka, T. Pagler, and N. Wang, “HDL, ABC Transporters, and Cholesterol Efflux: Implications for the Treatment of Atherosclerosis,” *Cell Metab.*, vol. 7, no. 5, pp. 365–375, May 2008.
[199] M. P. Adorni *et al.*, “The roles of different pathways in the release of cholesterol from macrophages,” *J. Lipid Res.*, vol. 48, no. 11, pp. 2453–2462, Nov. 2007.
[200] N. Rotllan, V. Ribas, L. Calpe-Berdiel, J. M. Martín-Campos, F. Blanco-Vaca, and J. C. Escolà-Gil, “Overexpression of Human Apolipoprotein A-II in Transgenic Mice Does Not Impair Macrophage-Specific Reverse Cholesterol Transport In Vivo,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 25, no. 9, pp. e128-32, Sep. 2005.
[201] A. Tricerri, B. Córsico, J. D. Toledo, H. A. Garda, and R. R. Brenner, “Conformation of apolipoprotein AI in reconstituted lipoprotein particles and particle-membrane interaction: effect of cholesterol.,” *Biochim. Biophys. Acta*, vol. 1391, no. 1, pp. 67–78, Mar. 1998.
[202] Y. Zhang, I. Zanotti, M. P. Reilly, J. M. Glick, G. H. Rothblat, and D. J. Rader, “Overexpression of Apolipoprotein A-I Promotes Reverse Transport of Cholesterol From Macrophages to Feces In Vivo,” *Circulation*, vol. 108, no. 6, pp. 661–663, Aug. 2003.
[203] J. M. Timmins *et al.*, “Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I,” *J. Clin. Invest.*, vol. 115, no. 5, pp. 1333–1342, May 2005.
[204] M. Efrat and M. Aviram, “Paraoxonase 1 Interactions with HDL, Antioxidants and Macrophages Regulate Atherogenesis – A Protective Role for HDL Phospholipids,” in *Advances in experimental medicine and biology*, vol. 660, 2010, pp. 153–166.
[205] G. J. Koelwyn, E. M. Corr, E. Erbay, and K. J. Moore, “Regulation of macrophage immunometabolism in atherosclerosis,” *Nat. Immunol.*, vol. 19, no. 6, pp. 526–537, Jun. 2018.
[206] W. S. Davidson, R. A. G. D. Silva, S. Chantepie, W. R. Lagor, M. J. Chapman, and A. Kontush, “Proteomic Analysis of Defined HDL Subpopulations Reveals Particle-Specific Protein Clusters,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 29, no. 6, pp. 870–876, Jun. 2009.
[207] M. Navab, S. T. Reddy, B. J. Van Lenten, G. M. Anantharamaiah, and A. M. Fogelman, “The role of dysfunctional HDL in atherosclerosis,” *J. Lipid Res.*, vol. 50, no. Supplement, pp. S145–S149, Apr. 2009.
[208] M. Navab *et al.*, “Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1.,” *J. Lipid Res.*, vol. 41, no. 9, pp. 1481–94, Sep. 2000.
[209] M. J. Stampfer, F. M. Sacks, S. Salvini, W. C. Willett, and C. H. Hennekens, “A Prospective Study of Cholesterol, Apolipoproteins, and the Risk of Myocardial Infarction,” *N. Engl. J. Med.*, vol. 325, no. 6, pp. 373–381, Aug. 1991.
[210] B. F. Asztalos *et al.*, “Value of High-Density Lipoprotein (HDL) Subpopulations in Predicting Recurrent Cardiovascular Events in the Veterans Affairs HDL Intervention Trial,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 25, no. 10, pp. 2185–2191, Oct. 2005.

[211] B. J. Van Lenten, A. C. Wagner, D. P. Nayak, S. Hama, M. Navab, and A. M. Fogelman, “High-density lipoprotein loses its anti-inflammatory properties during acute influenza a infection.,” *Circulation*, vol. 103, no. 18, pp. 2283–8, May 2001.
[212] G. S. Getz and C. A. Reardon, “SAA, HDL biogenesis, and inflammation,” *J. Lipid Res.*, vol. 49, no. 2, pp. 269–270, Feb. 2008.
[213] R. S. Rosenson *et al.*, “Dysfunctional HDL and atherosclerotic cardiovascular disease,” *Nat Rev Cardiol*, vol. 13, no. 1, pp. 48–60, 2016.
[214] M. Cuchel and D. J. Rader, “Macrophage reverse cholesterol transport: Key to the regression of atherosclerosis?,” *Circulation*, vol. 113, no. 21, pp. 2548–2555, 2006.
[215] J. Nimpf and W. J. Schneider, “From cholesterol transport to signal transduction: Low density lipoprotein receptor, very low density lipoprotein receptor, and apolipoprotein E receptor-2,” *Biochim. Biophys. Acta-Molecular Cell Biol. Lipids*, vol. 1529, no. 1–3, pp. 287–298, 2000.
[216] S. Yamashita, K. ichi Hirano, N. Sakai, and Y. Matsuzawa, “Molecular biology and pathophysiological aspects of plasma cholesteryl ester transfer protein,” *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, vol. 1529, no. 1–3, pp. 257–275, 2000.
[217] J. F. Oram and A. M. Vaughan, “ATP-binding cassette cholesterol transporters and cardiovascular disease,” *Circ. Res.*, vol. 99, no. 10, pp. 1031–1043, 2006.
[218] T.-Y. Chang, C. C. Y. Chang, N. Ohgami, and Y. Yamauchi, “Cholesterol Sensing, Trafficking, and Esterification,” *Annu. Rev. Cell Dev. Biol.*, vol. 22, no. 1, pp. 129–157, 2006.
[219] E. Ikonen, “Cellular cholesterol trafficking and compartmentalization,” *Nat. Rev. Mol. Cell Biol.*, vol. 9, no. 2, pp. 125–138, 2008.
[220] R. S. Rosenson *et al.*, “Cholesterol efflux and atheroprotection: Advancing the concept of reverse cholesterol transport,” *Circulation*, vol. 125, no. 15, pp. 1905–1919, doi:10.1161/CIRCULATIONAHA.111.066589, 2012.
[221] D. Wang *et al.*, “Cyanidin-3-O-β-glucoside upregulates hepatic cholesterol 7α-hydroxylase expression and reduces hypercholesterolemia in mice,” *Mol. Nutr. Food Res.*, vol. 56, no. 4, pp. 610–621, 2012.
[222] K. J. Moore, F. J. Sheedy, and E. A. Fisher, “Macrophages in atherosclerosis: A dynamic balance,” *Nat. Rev. Immunol.*, vol. 13, no. 10, pp. 709–721, Sep. 2013.
[223] J. Kzhyshkowska, C. Neyen, and S. Gordon, “Role of macrophage scavenger receptors in atherosclerosis,” *Immunobiology*, vol. 217, no. 5, pp. 492–502, 2012.
[224] C. Xie *et al.*, “Lowbush blueberries inhibit scavenger receptors CD36 and SR-A expression and attenuate foam cell formation in ApoE-deficient mice,” *Food Funct.*, vol. 2, no. 10, pp. 588–594, 2011.
[225] J. Canton, D. Neculai, and S. Grinstein, “Scavenger receptors in homeostasis and immunity,” *Nat Rev Immunol*, vol. 13, no. 9, pp. 621–634, 2013.
[226] Y. Wang, Y. Zhang, X. Wang, Y. Liu, and M. Xia, “Supplementation with cyanidin-3-O-beta-glucoside protects against hypercholesterolemia-mediated endothelial dysfunction and attenuates atherosclerosis in apolipoprotein E-deficient mice,” *J Nutr*, vol. 142, no. 6, pp. 1033–1037, 2012.
[227] C. Wu *et al.*, “Chlorogenic acid protects against atherosclerosis in ApoE-/-mice and promotes cholesterol efflux from RAW264.7 macrophages,” *PLoS One*, vol. 9, no. 9, p. e95452, 2014.
[228] D. Wang *et al.*, “Gut microbiota metabolism of anthocyanin promotes reverse cholesterol transport in mice via repressing miRNA-10b,” *Circ. Res.*, vol. 111, no. 8, pp. 967–981, Sep. 2012.
[229] E. Van Rymenant *et al.*, “Vasorelaxant activity of twenty-one physiologically relevant (poly)phenolic metabolites on isolated mouse arteries.,” *Food Funct.*,

vol. 8, no. 12, pp. 4331–4335, doi:10.1039/c7fo01273j, Dec. 2017.
[230] G. M. Woodward, P. W. Needs, and C. D. Kay, “Anthocyanin-derived phenolic acids form glucuronides following simulated gastrointestinal digestion and microsomal glucuronidation,” *Mol. Nutr. Food Res.*, vol. 55, no. 3, pp. 378–386, 2011.
[231] K. Goszcz, S. J. Deakin, G. G. Duthie, D. Stewart, and I. L. Megson, “Bioavailable Concentrations of Delphinidin and Its Metabolite, Gallic Acid, Induce Antioxidant Protection Associated with Increased Intracellular Glutathione in Cultured Endothelial Cells,” *Oxid. Med. Cell. Longev.*, vol. 2017, pp. 1–17, 2017.
[232] J. M. Hodgson, L. W. Morton, I. B. Puddey, L. J. Beilin, and K. D. Croft, “Gallic Acid Metabolites Are Markers of Black Tea Intake in Humans,” 2000.
[233] T. Nurmi, J. Mursu, M. Heinonen, A. Nurmi, R. Hiltunen, and S. Voutilainen, “Metabolism of berry anthocyanins to phenolic acidsin humans,” *J. Agric. Food Chem.*, vol. 57, no. 6, pp. 2274–2281, 2009.
[234] P. E. L. Day, K. F. Chambers, M. S. Winterbone, T. García-Blanco, D. Vauzour, and P. A. Kroon, “Validation of control genes and a standardised protocol for quantifying gene expression in the livers of C57BL/6 and ApoE−/− mice,” *Sci. Rep.*, vol. 8, no. 1, p. 8081, Dec. 2018.
[235] M. Rosenblat, J. Vaya, D. Shih, and M. Aviram, “Paraoxonase 1 (PON1) enhances HDL-mediated macrophage cholesterol efflux via the ABCA1 transporter in association with increased HDL binding to the cells: A possible role for lysophosphatidylcholine,” *Atherosclerosis*, vol. 179, no. 1, pp. 69–77, 2005.
[236] P. Zheng, A. Horwitz, C. A. Waelde, and J. D. Smith, “Stably transfected ABCA1 antisense cell line has decreased ABCA1 mRNA and cAMP-induced cholesterol efflux to apolipoprotein AI and HDL,” *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, vol. 1534, no. 2–3, pp. 121–128, Dec. 2001.
[237] J. F. Oram, R. M. Lawn, M. R. Garvin, and D. P. Wade, “ABCA1 Is the cAMP-inducible Apolipoprotein Receptor That Mediates Cholesterol Secretion from Macrophages,” *J. Biol. Chem.*, vol. 275, no. 44, pp. 34508–34511, Nov. 2000.
[238] L. Kritharides, A. Christian, G. Stoudt, D. Morel, and G. H. Rothblat, “Cholesterol metabolism and efflux in human THP-1 macrophages,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 18, no. 10, pp. 1589–1599, 1998.
[239] Z. Wei, A. O. Batagov, D. R. F. Carter, and A. M. Krichevsky, “Fetal Bovine Serum RNA Interferes with the Cell Culture derived Extracellular RNA,” *Sci. Rep.*, vol. 6, p. 31175, 2016.
[240] H. Cao, X. Liu, N. P. Ulrih, P. K. Sengupta, and J. Xiao, “Plasma protein binding of dietary polyphenols to human serum albumin: A high performance affinity chromatography approach,” *Food Chem.*, vol. 270, pp. 257–263, doi:10.1016/j.foodchem.2018.07.111, Jan. 2019.
[241] N. Latruffe, M. Menzel, D. Delmas, R. Buchet, and A. Lançon, “Compared Binding Properties between Resveratrol and Other Polyphenols to Plasmatic Albumin: Consequences for the Health Protecting Effect of Dietary Plant Microcomponents,” *Molecules*, vol. 19, no. 11, pp. 17066–17077, doi:10.3390/molecules191117066, Oct. 2014.
[242] A. Nozaki, M. Hori, T. Kimura, H. Ito, and T. Hatano, “Interaction of Polyphenols with Proteins: Binding of (−)-Epigallocatechin Gallate to Serum Albumin, Estimated by Induced Circular Dichroism,” *Chem. Pharm. Bull. (Tokyo).*, vol. 57, no. 2, pp. 224–228, doi:10.1248/cpb.57.224, Feb. 2009.
[243] D. A. Chistiakov, A. A. Melnichenko, V. A. Myasoedova, A. V. Grechko, and A. N. Orekhov, “Mechanisms of foam cell formation in atherosclerosis,” *J. Mol. Med.*, vol. 95, no. 11, pp. 1153–1165, Nov. 2017.
[244] M. Menju, S. Tajima, and A. Yamamoto, “Expression of the Apolipoprotein E Gene in a Human Macrophage-Like Cell Line, THP-1,” *J. Biochem.*, vol. 106,

no. 3, pp. 505–510, Sep. 1989.
[245] S. Tsuchiya, M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada, “Establishment and characterization of a human acute monocytic leukemia cell line (THP-1).,” *Int. J. cancer*, vol. 26, no. 2, pp. 171–6, Aug. 1980.
[246] I. Zani *et al.*, “Scavenger Receptor Structure and Function in Health and Disease,” *Cells*, vol. 4, no. 2, pp. 178–201, 2015.
[247] A. Ji *et al.*, “Scavenger receptor SR-BI in macrophage lipid metabolism,” *Atherosclerosis*, vol. 217, no. 1, pp. 106–112, 2011.
[248] R. L. Silverstein, W. Li, S. Ohidar, and R. Cleveland, “Mechanisms of Cell Signaling By the Scavenger Receptor Cd36: Implications in Atherosclerosis and Thrombosis,” *Trans. Am. Clin. Climatol. Assoc.*, vol. 121, pp. 206–220, 2010.
[249] I. Krga *et al.*, “Anthocyanins and their gut metabolites attenuate monocyte adhesion and transendothelial migration through nutrigenomic mechanisms regulating endothelial cell permeability,” *Free Radic. Biol. Med.*, vol. 124, pp. 364–379, Aug. 2018.
[250] H. P. Amin *et al.*, “Anthocyanins and their physiologically relevant metabolites alter the expression of IL-6 and VCAM-1 in CD40L and oxidized LDL challenged vascular endothelial cells,” *Mol. Nutr. Food Res.*, vol. 59, no. 6, pp. 1095–1106, doi:10.1002/mnfr.201400803, 2015.
[251] M. Y. Um, J. Ahn, and T. Y. Ha, “Hypolipidaemic effects of cyanidin 3-glucoside rich extract from black rice through regulating hepatic lipogenic enzyme activities,” *J. Sci. Food Agric.*, vol. 93, no. 12, pp. 3126–3128, 2013.
[252] D. Wang *et al.*, “Cyanidin-3-O-beta-glucoside upregulates hepatic cholesterol 7alpha-hydroxylase expression and reduces hypercholesterolemia in mice,” *Mol Nutr Food Res*, vol. 56, no. 4, pp. 610–621, 2012.
[253] S. Ikhlef, H. Berrougui, O. K. Simo, E. Zerif, and A. Khalil, “Human paraoxonase 1 overexpression in mice stimulates HDL cholesterol efflux and reverse cholesterol transport,” *PLoS One*, vol. 12, no. 3, p. doi:10.1371/journal.pone.0173385, 2017.
[254] A. Mahrooz, M.-R. Rashidi, and M. Nouri, “Naringenin is an inhibitor of human serum paraoxonase (PON1): an in vitro study,” *J. Clin. Lab. Anal.*, vol. 25, no. 6, pp. 395–401, doi:10.1002/jcla.20490, Nov. 2011.
[255] S. K. Patra, K. Singh, and R. Singh, “Paraoxonase 1: A better atherosclerotic risk predictor than HDL in type 2 diabetes mellitus,” *Diabetes Metab. Syndr. Clin. Res. Rev.*, vol. 7, no. 2, pp. 108–111, 2013.
[256] A. Gugliucci and T. Menini, “Paraoxonase 1 and HDL maturation,” *Clin. Chim. Acta*, vol. 439, pp. 5–13, doi:10.1016/j.cca.2014.09.016, 2015.
[257] S. Loued, H. Berrougui, P. Componova, S. Ikhlef, O. Helal, and A. Khalil, “Extra-virgin olive oil consumption reduces the age-related decrease in HDL and Paraoxonase 1 anti-inflammatory activities,” *Br. J. Nutr.*, vol. 110, no. 7, pp. 1272–1284, 2013.
[258] S. Deakin, I. Leviev, S. Guernier, and R. W. James, “Simvastatin Modulates Expression of the PON1 Gene and Increases Serum Paraoxonase: A Role for Sterol Regulatory Element-Binding Protein-2,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 23, no. 11, pp. 2083–2089, 2003.
[259] K. Sumegová *et al.*, “Activity of Paraoxonase 1 and Lipid Profile in Healthy Children,” *Physiol. Res*, vol. 56, no. 3, pp. 351–357, 2007.
[260] J. J. Ceron, F. Tecles, and A. Tvarijonaviciute, “Serum paraoxonase 1 (PON1) measurement: an update,” *BMC Vet Res*, vol. 10, pp. 74, doi:10.1186/1746-6148-10–74, 2014.
[261] A. I. Rupérez *et al.*, “Paraoxonase 1 activities and genetic variation in childhood obesity,” *Br. J. Nutr.*, vol. 110, no. 9, pp. 1639–1647, doi:10.1017/s0007114513001967, 2013.
[262] M. Rosenblat *et al.*, “The catalytic histidine dyad of high density lipoprotein-

associated serum paraoxonase-1 (PON1) is essential for PON1-mediated inhibition of low density lipoprotein oxidation and stimulation of macrophage cholesterol efflux," *J. Biol. Chem.*, vol. 281, no. 11, pp. 7657–7665, doi:10.1074/jbc.M512595200, 2006.

[263] X. Yang *et al.*, "Plasma homocysteine thiolactone adducts associated with risk of coronary heart disease," *Clin. Chim. Acta*, vol. 364, no. 1–2, pp. 230–234, 2006.

[264] N. Gupta, S. Singh, V. N. Maturu, Y. P. Sharma, and K. D. Gill, "Paraoxonase 1 (PON1) polymorphisms, haplotypes and activity in predicting CAD risk in North-West Indian Punjabis," *PLoS One*, vol. 6, no. 5, pp. e17805, doi:10.1371/journal.pone.0017805, 2011.

[265] B. Mackness *et al.*, "Serum paraoxonase (PON1) 55 and 192 polymorphism and paraoxonase activity and concentration in non-insulin dependent diabetes mellitus," *Atherosclerosis*, vol. 139, no. 2, pp. 341–349, doi:10.1016/S0021-9150(98)00095–1, 1998.

[266] A. Lesions *et al.*, "Human Serum Paraoxonases (PON1) Q and R Selectively Decrease Lipid Peroxides in Human Coronary and Carotid," *Circulation*, vol. 101, no. 21, pp. 2510–2518, 2000.

[267] E. E. Quillen *et al.*, "Novel Associations of Nonstructural Loci with Paraoxonase Activity," *J. Lipids*, vol. 2012, pp. 1–7, doi:10.1155/2012/189681, 2012.

[268] V. H. Brophy, M. D. Hastings, J. B. Clendenning, R. J. Richter, G. P. Jarvik, and C. E. Furlong, "Polymorphisms in the human paraoxonase (PON1) promoter," *Pharmacogenetics*, vol. 11, no. 1, pp. 77–84, doi:10.1097/00008571-200102000–00009, 2001.

[269] L. ME *et al.*, "A functional polymorphism of PON1 interferes with microRNA binding to increase the risk of ischemic stroke and carotid atherosclerosis.," *Atherosclerosis*, vol. 228, no. 1, pp. 161–167, doi:10.1016/j.atherosclerosis.2013.01.036, 2013.

[270] F. G. Santos *et al.*, "The effect of the paraoxonase 1 (PON1) T(-107)C polymorphism on serum PON1 activity in women is dependent on fatty acid intake," *Nutr. Res.*, vol. 36, no. 1, pp. 9–15, doi:10.1016/j.nutres.2015.10.008, 2016.

[271] H. Soran, N. N. Younis, V. Charlton-Menys, and P. Durrington, "Variation in paraoxonase-1 activity and atherosclerosis," *Curr. Opin. Lipidol.*, vol. 20, no. 4, pp. 265–274, 2009.

[272] G. P. Jarvik *et al.*, "Vitamin C and E intake is associated with increased paraoxonase activity," *Arterioscler. Thromb. Vasc. Biol.*, vol. 22, no. 8, pp. 1329–1333, 2002.

[273] C. C. Federica Rizzi Elena Dogliotti, Annalisa Terranegra, Erika Salvi, Daniele Braga, Flavia Ricca, Sara Lupoli, Alessandra Mingione, Francesca Pivari, Caterina Brasacchio, Matteo Barcella, Martina Chittani, Francesca D'Avila, Maurizio Turiel, Monica Lazzaron *et al.*, "Interaction between polyphenols intake and PON1 gene variants on markers of cardiovascular disease: a nutrigenetic observational study," *J Transl Med*, vol. 14, no. 1, pp. 10, doi:10.1186/s12967-016-0941–6, 2016.

[274] B. Fuhrman *et al.*, "Pomegranate juice consumption reduces oxidative stress and low density lipoprotein atherogenic modifications: studies in the atherosclerotic apolipoprotein E deficient mice and in humans," *Atherosclerosis*, vol. 151, no. 1, p. 111, Jul. 2000.

[275] C. Boesch-Saadatmandi *et al.*, "Effect of quercetin on paraoxonase 1 activity - studies in cultured cells, mice and humans," *J. Physiol. Pharmacol.*, vol. 61, no. 1, pp. 99–105, 2010.

[276] B. Fuhrman and M. Aviram, "Preservation of paraoxonase activity by wine flavonoids: Possible role in protection of LDL from lipid peroxidation," *Ann. N. Y. Acad. Sci.*, vol. 957, pp. 321–324, doi:10.1111/j.1749-6632.2002.tb02933.x,

2002.
[277] B. Ustundag *et al.*, “Protective effect of soy isoflavones and activity levels of plasma paraoxonase and arylesterase in the experimental nonalcoholic steatohepatitis model,” *Dig Dis Sci*, vol. 52, no. 8, pp. 2006–2014, doi:10.1007/s10620-006-9251–9, 2007.
[278] T. Esatbeyoglu, M. Rodríguez-Werner, A. Schlösser, P. Winterhalter, and G. Rimbach, “Fractionation, enzyme inhibitory and cellular antioxidant activity of bioactives from purple sweet potato (Ipomoea batatas),” *Food Chem.*, vol. 221, pp. 447–456, doi:10.1016/J.FOODCHEM.2016.10.077, Apr. 2017.
[279] C. Gouedard, R. Barouki, Y. Morel, C. Gouédard, R. Barouki, and Y. Morel, “Induction of the paraoxonase-1 gene expression by resveratrol,” *Arter. Thromb Vasc Biol*, vol. 24, no. 12, pp. 2378–2383, doi:10.1161/01.ATV.0000146530.24736.ce, 2004.
[280] N. Gupta, R. Kandimalla, K. Priyanka, G. Singh, K. D. Gill, and S. Singh, “Effect of resveratrol and nicotine on PON1 gene expression: In vitro study,” *Indian J. Clin. Biochem.*, vol. 29, no. 1, pp. 69–73, doi:10.1007/s12291-013-0300–9, 2014.
[281] C. Gouedard *et al.*, “Dietary polyphenols increase paraoxonase 1 gene expression by an aryl hydrocarbon receptor-dependent mechanism,” *Mol Cell Biol*, vol. 24, no. 12, pp. 5209–5222, doi:10.1128/mcb.24.12.5209-5222.2004, Jun. 2004.
[282] C. Schrader, C. Schiborr, J. Frank, and G. Rimbach, “Curcumin induces paraoxonase 1 in cultured hepatocytes in vitro but not in mouse liver in vivo,” *Br. J. Nutr.*, vol. 105, no. 2, pp. 167–170, doi:10.1017/S0007114510004356, 2011.
[283] P. Jaichander, K. Selvarajan, M. Garelnabi, and S. Parthasarathy, “Induction of paraoxonase 1 and apolipoprotein A-I gene expression by aspirin,” *J. Lipid Res.*, vol. 49, no. 10, pp. 2142–2148, doi:10.1194/jlr.M800082-JLR200, Oct. 2008.
[284] A. Tvarijonaviciute, F. Tecles, M. Caldin, S. Tasca, and J. Cerón, “Validation of spectrophotometric assays for serum paraoxonase type-1 measurement in dogs,” *Am. J. Vet. Res.*, vol. 73, no. 1, pp. 34–41, doi:10.2460/ajvr.73.1.34, 2012.
[285] R. W. Browne, S. T. Koury, S. Marion, G. Wilding, P. Muti, and M. Trevisan, “Accuracy and biological variation of human serum paraoxonase 1 activity and polymorphism (Q192R) by kinetic enzyme assay,” *Clin. Chem.*, vol. 53, no. 2, pp. 310–317, doi:10.1373/clinchem.2006.074559, 2007.
[286] N. Martinelli *et al.*, “Novel serum paraoxonase activity assays are associated with coronary artery disease,” *Clin. Chem. Lab. Med.*, vol. 47, no. 4, pp. 432–440, doi:10.1515/CCLM.2009.108, 2009.
[287] L. Gaidukov and D. S. Tawfik, “The development of human sera tests for HDL-bound serum PON1 and its lipolactonase activity,” *J. Lipid Res.*, vol. 48, no. 7, pp. 1637–1646, doi:10.1194/jlr.D600045-JLR200, 2007.
[288] O. Khersonsky and D. S. Tawfik, “Chromogenic and fluorogenic assays for the lactonase activity of serum paraoxonases,” *ChemBioChem*, vol. 7, no. 1, pp. 49–53, doi:10.1002/cbic.200500334, 2006.
[289] N. Ferre *et al.*, “Impaired paraoxonase-1 status in obese children. Relationships with insulin resistance and metabolic syndrome,” *Clin Biochem*, vol. 46, no. 18, pp. 1830–1836, doi:10.1016/j.clinbiochem.2013.08.020, 2013.
[290] T. Esatbeyoglu, K. Ulbrich, C. Rehberg, S. Rohn, and G. Rimbach, “Thermal stability, antioxidant, and anti-inflammatory activity of curcumin and its degradation product 4-vinyl guaiacol,” *Food Funct.*, vol. 6, no. 3, pp. 887–893, doi:10.1039/c4fo00790e, 2015.
[291] J. Khateeb, A. Gantman, A. J. Kreitenberg, M. Aviram, and B. Fuhrman, “Paraoxonase 1 (PON1) expression in hepatocytes is upregulated by

pomegranate polyphenols: A role for PPAR-gamma pathway," *Atherosclerosis*, vol. 208, no. 1, pp. 119–125, doi:10.1016/j.atherosclerosis.2009.08.051, Feb. 2010.
[292] M. Garige, M. Gong, R. Varatharajalu, and M. R. Lakshman, "Quercetin up-regulates paraoxonase 1 gene expression via sterol regulatory element binding protein 2 that translocates from the endoplasmic reticulum to the nucleus where it specifically interacts with sterol responsive element-like sequence in paraoxonas," *Metabolism.*, vol. 59, no. 9, pp. 1372–1378, 2010.
[293] B. Gokce, N. Gencer, O. Arslan, M. O. Karatas, and B. Alici, "In vitro inhibition effect of some coumarin compounds on purified human serum paraoxonase 1 (PON1)," *J. Enzyme Inhib. Med. Chem.*, vol. 31, no. 4, pp. 534–537, 2016.
[294] T. L. Graves and J. E. Scott, "A High Throughput Serum Paraoxonase Assay for Discovery of Small Molecule Modulators of PON1 Activity," *Curr. Chem. Genomics*, vol. 2, pp. 51–61, doi:10.2174/1875397300802010051, 2008.
[295] D. Levy, C. O. Reichert, S. P. Bydlowski, D. Levy, C. O. Reichert, and S. P. Bydlowski, "Paraoxonases activities and polymorphisms in elderly and old-age diseases: An overview," *Antioxidants*, vol. 8, no. 5, pp. 118, doi:10.3390/antiox8050118, May 2019.
[296] W. Phuntuwate, C. Suthisisang, B. Koanantakul, M. I. Mackness, and B. Mackness, "Paraoxonase 1 status in the Thai population," *J. Hum. Genet.*, vol. 50, no. 6, pp. 293–300, doi:10.1007/s10038-005-0255–7, 2005.
[297] D. Atrahimovich, J. Vaya, H. Tavori, and S. Khatib, "Glabridin protects paraoxonase 1 from linoleic acid hydroperoxide inhibition via specific interaction: A fluorescence-quenching study," *J. Agric. Food Chem.*, vol. 60, no. 14, pp. 3679–3685, doi:10.1021/jf2046009, 2012.
[298] S. Deakin, I. Leviev, M. Gomaraschi, L. Calabresi, G. Franceschini, and R. W. James, "Enzymatically active paraoxonase-1 is located at the external membrane of producing cells and released by a high affinity, saturable, desorption mechanism," *J. Biol. Chem.*, vol. 277, no. 6, pp. 4301–4308, doi:10.1074/jbc.M107440200, Feb. 2002.
[299] C. Yuan, "Techniques and Applications of Atherosclerosis MRI," 2010.
[300] N. Katakami, H. Kaneto, and I. Shimomura, "Carotid ultrasonography: A potent tool for better clinical practice in diagnosis of atherosclerosis in diabetic patients," *J. Diabetes Investig.*, vol. 5, no. 1, pp. 3–13, Jan. 2014.
[301] A. M. Amini, K. Muzs, J. P. Spencer, and P. Yaqoob, "Pelargonidin-3-O-glucoside and its metabolites have modest anti-inflammatory effects in human whole blood cultures," *Nutr. Res.*, vol. 46, pp. 88–95, 2017.
[302] A. M. Amini, J. P. E. Spencer, and P. Yaqoob, "Effects of pelargonidin-3-O-glucoside and its metabolites on lipopolysaccharide-stimulated cytokine production by THP-1 monocytes and macrophages," *Cytokine*, vol. 103, pp. 29–33, 2018.
[303] I. Krga *et al.*, "Anthocyanins and their gut metabolites attenuate monocyte adhesion and transendothelial migration through nutrigenomic mechanisms regulating endothelial cell permeability," *Free Radic Biol Med*, vol. 124, pp. 364–379, 2018.
[304] S. Kakkar and S. Bais, "A Review on Protocatechuic Acid and Its Pharmacological Potential," *ISRN Pharmacol.*, vol. 2014, pp. 1–9, 2014.
[305] X. Liu *et al.*, "Transcriptomic analysis of the response of Pseudomonas fluorescens to epigallocatechin gallate by RNA-seq," *PLoS One*, vol. 12, no. 5, p. e0177938, May 2017.
[306] S. Backman *et al.*, "RNA Sequencing Provides Novel Insights into the Transcriptome of Aldosterone Producing Adenomas," *Sci. Rep.*, vol. 9, no. 1, p. 6269, Dec. 2019.
[307] A. Rodriguez-Mateos *et al.*, "Circulating anthocyanin metabolites mediate vascular benefits of blueberries: insights from randomized controlled trials,

metabolomics, and nutrigenomics," *Journals Gerontol. Ser. A*, Feb. 2019.
[308] A. Mauray, C. Felgines, C. Morand, A. Mazur, A. Scalbert, and D. Milenkovic, "Nutrigenomic analysis of the protective effects of bilberry anthocyanin-rich extract in apo E-deficient mice," *Genes Nutr.*, vol. 5, no. 4, pp. 343–353, 2010.
[309] J. Chen, T. Uto, S. Tanigawa, T. Kumamoto, M. Fujii, and D.-X. Hou, "Expression Profiling of Genes Targeted by Bilberry (*Vaccinium myrtillus*) in Macrophages Through DNA Microarray," *Nutr. Cancer*, vol. 60, no. sup1, pp. 43–50, Nov. 2008.
[310] T. Tsuda, Y. Ueno, T. Yoshikawa, H. Kojo, and T. Osawa, "Microarray profiling of gene expression in human adipocytes in response to anthocyanins," *Biochem. Pharmacol.*, vol. 71, no. 8, pp. 1184–1197, Apr. 2006.
[311] T. Tsuda, Y. Ueno, H. Kojo, T. Yoshikawa, and T. Osawa, "Gene expression profile of isolated rat adipocytes treated with anthocyanins," *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, vol. 1733, no. 2–3, pp. 137–147, Apr. 2005.
[312] S. Zhao, W.-P. Fung-Leung, A. Bittner, K. Ngo, and X. Liu, "Comparison of RNA-Seq and Microarray in Transcriptome Profiling of Activated T Cells," *PLoS One*, vol. 9, no. 1, p. e78644, Jan. 2014.
[313] B. Li *et al.*, "Transcriptome Analysis of Psoriasis in a Large Case–Control Sample: RNA-Seq Provides Insights into Disease Mechanisms," *J. Invest. Dermatol.*, vol. 134, no. 7, pp. 1828–1838, Jul. 2014.
[314] E. de Klerk and P. A. C. 't Hoen, "Alternative mRNA transcription, processing, and translation: insights from RNA sequencing," *Trends Genet.*, vol. 31, no. 3, pp. 128–139, Mar. 2015.
[315] M. F. Rai, E. D. Tycksen, L. J. Sandell, and R. H. Brophy, "Advantages of RNA-seq compared to RNA microarrays for transcriptome profiling of anterior cruciate ligament tears," *J. Orthop. Res.*, Aug. 2017.
[316] M. Pertea, D. Kim, G. M. Pertea, J. T. Leek, and S. L. Salzberg, "Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown," *Nat. Protoc.*, vol. 11, no. 9, pp. 1650–1667, Sep. 2016.
[317] E. Kopylova, L. Noé, and H. Touzet, "SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data," *Bioinformatics*, vol. 28, no. 24, pp. 3211–3217, Dec. 2012.
[318] D. Kim, B. Langmead, and S. L. Salzberg, "HISAT: a fast spliced aligner with low memory requirements," *Nat. Methods*, vol. 12, no. 4, pp. 357–360, Apr. 2015.
[319] M. Pertea, G. M. Pertea, C. M. Antonescu, T.-C. Chang, J. T. Mendell, and S. L. Salzberg, "StringTie enables improved reconstruction of a transcriptome from RNA-seq reads," *Nat. Biotechnol.*, vol. 33, no. 3, pp. 290–295, Mar. 2015.
[320] M. D. Robinson, D. J. McCarthy, and G. K. Smyth, "edgeR: a Bioconductor package for differential expression analysis of digital gene expression data," *Bioinformatics*, vol. 26, no. 1, pp. 139–140, Jan. 2010.
[321] D. J. McCarthy, Y. Chen, and G. K. Smyth, "Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation.," *Nucleic Acids Res.*, vol. 40, no. 10, pp. 4288–97, May 2012.
[322] M. E. Ritchie *et al.*, "limma powers differential expression analyses for RNA-sequencing and microarray studies," *Nucleic Acids Res.*, vol. 43, no. 7, pp. e47–e47, Apr. 2015.
[323] D. R. Zerbino *et al.*, "Ensembl 2018," *Nucleic Acids Res.*, vol. 46, no. D1, pp. D754–D761, Jan. 2018.
[324] A. Mortazavi, B. A. Williams, K. McCue, L. Schaeffer, and B. Wold, "Mapping and quantifying mammalian transcriptomes by RNA-Seq," *Nat. Methods*, vol. 5, no. 7, pp. 621–628, Jul. 2008.
[325] L. Cheng, X. Zhang, X. Zheng, Z. Wu, and P. Weng, "RNA-seq transcriptomic analysis of green tea polyphenols regulation of differently expressed genes in Saccharomyces cerevisiae under ethanol stress," *World J. Microbiol.*

Biotechnol., vol. 35, no. 4, p. 59, Apr. 2019.
[326] C. W. Law *et al.*, “RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR,” *F1000Research*, vol. 5, p. 1408, Dec. 2018.
[327] G. Fantuzzi and T. Mazzone, “Adipose tissue and atherosclerosis: exploring the connection.,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 27, no. 5, pp. 996–1003, May 2007.
[328] C. Bouvard *et al.*, “Small molecule selectively suppresses MYC transcription in cancer cells.”
[329] G. R. Stark and W. R. Taylor, “Analyzing the G2/M Checkpoint,” in *Checkpoint Controls and Cancer*, vol. 280, New Jersey: Humana Press, 2004, pp. 051–082.
[330] S. R. Smith and P. W. F. Wilson, “Free Fatty Acids and Atherosclerosis—Guilty or Innocent?,” *J. Clin. Endocrinol. Metab.*, vol. 91, no. 7, pp. 2506–2508, Jul. 2006.
[331] C. Souilhol, M. C. Harmsen, P. C. Evans, and G. Krenning, “Endothelial–mesenchymal transition in atherosclerosis,” *Cardiovasc. Res.*, vol. 114, no. 4, pp. 565–577, Mar. 2018.
[332] M. Wesseling, T. R. Sakkers, S. C. A. de Jager, G. Pasterkamp, and M. J. Goumans, “The morphological and molecular mechanisms of epithelial/endothelial-to-mesenchymal transition and its involvement in atherosclerosis,” *Vascul. Pharmacol.*, vol. 106, pp. 1–8, Jul. 2018.
[333] T. Taniguchi and A. Takaoka, “The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors.,” *Curr. Opin. Immunol.*, vol. 14, no. 1, pp. 111–6, Feb. 2002.
[334] R. Vittal *et al.*, “Gene expression changes induced by green tea polyphenol (À)-epigallocatechin-3-gallate in human bronchial epithelial 21BES cells analyzed by DNA microarray,” 2004.
[335] Z. Song, L. Guo, T. Liu, C. Lin, J. Wang, and X. Li, “Comparative RNA-Sequence Transcriptome Analysis of Phenolic Acid Metabolism in Salvia miltiorrhiza, a Traditional Chinese Medicine Model Plant.,” *Int. J. Genomics*, vol. 2017, p. 9364594, 2017.
[336] D. Atrahimovich, A. O. Samson, Y. Barsheshet, J. Vaya, S. Khatib, and E. Reuveni, “Genome-wide localization of the polyphenol quercetin in human monocytes,” *BMC Genomics*, vol. 20, no. 1, p. 606, Dec. 2019.
[337] M. Zhu *et al.*, “Integrated analysis of hepatic mRNA and miRNA profiles identified molecular networks and potential biomarkers of NAFLD,” *Sci. Rep.*, vol. 8, no. 1, p. 7628, Dec. 2018.
[338] M. Lefevre *et al.*, “Gene Expression Microarray Analysis of the Effects of Grape Anthocyanins in Mice-A Test of an Hypothesis Generating Paradigm.”
[339] S.-H. Son, Y.-H. Goo, B. H. Chang, and A. Paul, “Perilipin 2 (PLIN2)-Deficiency Does Not Increase Cholesterol-Induced Toxicity in Macrophages,” *PLoS One*, vol. 7, no. 3, p. e33063, Mar. 2012.
[340] A. P. Bracken, M. Ciro, A. Cocito, and K. Helin, “E2F target genes: unraveling the biology,” *Trends Biochem. Sci.*, vol. 29, no. 8, pp. 409–417, Aug. 2004.
[341] R. De Bruyn, R. Bollen, and F. Claessens, “Identification and Characterization of Androgen Response Elements,” in *Methods in molecular biology (Clifton, N.J.)*, vol. 776, 2011, pp. 81–93.
[342] C. E. Walczak and R. Heald, “Mechanisms of Mitotic Spindle Assembly and Function,” in *International review of cytology*, vol. 265, 2008, pp. 111–158.
[343] Z. Choudhry *et al.*, “Sonic hedgehog signalling pathway: a complex network.,” *Ann. Neurosci.*, vol. 21, no. 1, pp. 28–31, Jan. 2014.
[344] R. Kopan, “Notch signaling.,” *Cold Spring Harb. Perspect. Biol.*, vol. 4, no. 10, Oct. 2012.
[345] V. Moreno-Viedma *et al.*, “Common dysregulated pathways in obese adipose

tissue and atherosclerosis," *Cardiovasc. Diabetol.*, vol. 15, no. 1, p. 120, Dec. 2016.
[346] S. Harati, J. H. Phan, and M. D. Wang, "Investigation of factors affecting RNA-seq gene expression calls," in *2014 36th Annual International Conference of the IEEE Engineering in Medicine and Biology Society*, 2014, vol. 2014, pp. 5232–5235.
[347] S. Lamarre *et al.*, "Optimization of an RNA-Seq Differential Gene Expression Analysis Depending on Biological Replicate Number and Library Size," *Front. Plant Sci.*, vol. 9, p. 108, Feb. 2018.
[348] J. Z. B. Low, T. F. Khang, and M. T. Tammi, "CORNAS: coverage-dependent RNA-Seq analysis of gene expression data without biological replicates," *BMC Bioinformatics*, vol. 18, no. S16, p. 575, Dec. 2017.
[349] N. J. Schurch *et al.*, "How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use?," *RNA*, vol. 22, no. 6, pp. 839–51, 2016.
[350] W. J. Hollands *et al.*, "Lack of effect of bioactive-rich extracts of pomegranate, persimmon, nettle, dill, kale and Sideritis and isolated bioactives on platelet function."
[351] A. A. and and J. Strommer*, "A Simple Extraction and Chromatographic System for the Simultaneous Analysis of Anthocyanins and Stilbenes of Vitis Species," 2003.
[352] W. J. Hollands *et al.*, "Lack of acute or chronic effects of epicatechin-rich and procyanidin-rich apple extracts on blood pressure and cardiometabolic biomarkers in adults with moderately elevated blood pressure: A randomized, placebo-controlled crossover trial," *Am. J. Clin. Nutr.*, vol. 108, no. 5, pp. 1006–1014, 2018.
[353] K. Mori *et al.*, "Effects of abscisic acid treatment and night temperatures on anthocyanin composition in Pinot noir grapes," 2005.
[354] W. J. Hollands *et al.*, "Development, validation and evaluation of an analytical method for the determination of monomeric and oligomeric procyanidins in apple extracts," *J. Chromatogr. A*, vol. 1495, pp. 46–56, 2017.
[355] W. J. Hollands *et al.*, "Bioavailability of epicatechin and effects on nitric oxide metabolites of an apple flavanol-rich extract supplemented beverage compared to a whole apple puree: A randomized, placebo-controlled, crossover trial," *Mol. Nutr. Food Res.*, vol. 57, no. 7, pp. 1209–1217, 2013.
[356] W. J. Hollands *et al.*, "Development, validation and evaluation of an analytical method for the determination of monomeric and oligomeric procyanidins in apple extracts," *J. Chromatogr. A*, vol. 1495, pp. 46–56, 2017.
[357] L. Bao *et al.*, "Protective Effects of Bilberry (Vaccinium myrtillus L.) Extract on Restraint Stress-Induced Liver Damage in Mice," *J. Agric. Food Chem.*, vol. 56, no. 17, pp. 7803–7807, 2008.
[358] C. Ancillotti *et al.*, "Polyphenolic profiles and antioxidant and antiradical activity of Italian berries from Vaccinium myrtillus L. and Vaccinium uliginosum L. subsp. gaultherioides (Bigelow) S.B. Young," *Food Chem.*, vol. 204, pp. 176–184, 2016.
[359] X. Wu, L. Gu, R. L. Prior, and S. McKay, "Characterization of Anthocyanins and Proanthocyanidins in Some Cultivars of *Ribes* , *Aronia* , and *Sambucus* and Their Antioxidant Capacity," *J. Agric. Food Chem.*, 2004.
[360] S. Ahmad, J. J. Carter, and J. E. Scott, "A homogeneous cell-based assay for measurement of endogenous paraoxonase 1 activity," *Anal. Biochem.*, vol. 400, no. 1, pp. 1–9, May 2010.
[361] R. A. Sharstry, S. M. Biradar, K. M. Mahadevan, and P. V. Habbu, "Isolation and characterization of secondary metabolite from Amorphophallus paeoniifolius for hepatoprotective activity," *Res. J. Pharm. Biol. Chem. Sci.*, vol. 1, no. 4, pp. 429–437, 2010.

[362] D. B.F. *et al.*, “Association of genetic polymorphisms of PON1 and CETP with the presence of metabolic syndrome; the effects of genotypes on their serum activity and concentrations,” *Egypt. J. Med. Hum. Genet.*, vol. 19, no. 1, pp. 43–48, 2018.
[363] K. Huen *et al.*, “Associations of PON1 and genetic ancestry with obesity in early childhood,” *PLoS One*, vol. 8, no. 5, 2013.
[364] K. Huen *et al.*, “Developmental changes in PON1 enzyme activity in young children and effects of PON1 polymorphisms,” *Environ. Health Perspect.*, vol. 117, no. 10, pp. 1632–1638, 2009.
[365] D. Turgut Cosan *et al.*, “Association of paraoxonase 1 (PON1) gene polymorphisms and concentration with essential hypertension,” *Clin. Exp. Hypertens.*, vol. 38, no. 7, pp. 602–607, doi:10.3109/10641963.2016.1174255, Oct. 2016.
[366] S. Campo *et al.*, “Association between serum paraoxonase (PON1) gene promoter T(-107)C polymorphism, PON1 activity and HDL levels in healthy Sicilian octogenarians,” *Exp. Gerontol.*, vol. 39, no. 7, pp. 1089–1094, doi:10.1016/j.exger.2004.03.017, 2004.
[367] M. Grdic *et al.*, “Genetic frequencies of paraoxonase 1 gene polymorphisms in Croatian population,” *Croat. Chem. Acta*, vol. 81, no. 1, pp. 105–111, 2008.
[368] K. A. O’Leary *et al.*, “Genetic and other sources of variation in the activity of serum paraoxonase/diazoxonase in humans: consequences for risk from exposure to diazinon,” *Pharmacogenet. Genomics*, vol. 15, no. 1, pp. 51–60, 2005.
[369] L. G. Costa and C. E. Furlong, “Paraoxonase (PON1) in health aand disease: Basic and Clinical Aspects,” *Cardiol. Rev.*, vol. 15, no. 4, p. 216, 2002.
[370] V. H. Brophy, G. P. Jarvik, R. J. Richter, L. S. Rozek, G. D. Schellenberg, and C. E. Furlong, “Analysis of paraoxonase (PON1) L55M status requires both genotype and phenotype,” *Pharmacogenetics*, vol. 10, no. 5, pp. 453–460, 2000.
[371] G. Uliano, L. C. Muniz, C. C. Barros, A. Schneider, and S. C. Valle, “Association between paraoxonase 1 (PON1) enzyme activity, PON1 C(−107)T polymorphism, nutritional status, and lipid profile in children,” *Nutrire*, vol. 41, no. 1, p. 20, Dec. 2016.
[372] V. H. Brophy, R. L. Jampsa, J. B. Clendenning, L. A. McKinstry, and G. P. Jarvik, “Effects of 5’ regulatory-region polymorphisms on paraoxonase-gene (PON1) expression,” *Am J Hum Genet*, vol. 68, no. 6, pp. 1428–1436, doi:10.1086/320600 LB-Brophy2001, 2001.
[373] D. A. Lawlor *et al.*, “The association of the PON1 Q192R polymorphism with coronary heart disease: Findings from the British Women’s Heart and Health cohort study and a meta-analysis,” *BMC Genet.*, vol. 5, p. 17, 2004.
[374] W. S. Davidson *et al.*, “The effects of apolipoprotein B depletion on HDL subspecies composition and function,” *J. Lipid Res.*, vol. 57, no. 4, pp. 674–686, 2016.
[375] G. Walldius, I. Jungner, A. H. Aastveit, I. Holme, C. D. Furberg, and A. D. Sniderman, “The apoB/apoA-I ratio is better than the cholesterol ratios to estimate the balance between plasma proatherogenic and antiatherogenic lipoproteins and to predict coronary risk,” *Clin. Chem. Lab. Med.*, vol. 42, no. 12, pp. 1355–63, Jan. 2004.
[376] A. Skoczyñska *et al.*, “Influence of chokeberry juice on arterial blood pressure and lipid parameters in men with mild hypercholesterolemia,” *Pharmacol. reports*, vol. 59, no. SUPPL. 1, pp. 177–182, 2007.
[377] W. S. Davidson, R. A. G. D. Silva, S. Chantepie, W. R. Lagor, M. J. Chapman, and A. Kontush, “Proteomic analysis of defined hdl subpopulations reveals particle-specific protein clusters: Relevance to antioxidative function,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 29, no. 6, pp. 870–876, 2009.

[378] W. B. Kannel and D. L. McGee, “Diabetes and Glucose Tolerance as Risk Factors for Cardiovascular Disease: The Framingham Study,” *Diabetes Care*, vol. 2, no. 2, pp. 120–126, Mar. 1979.
[379] D. Li, Y. Zhang, Y. Liu, R. Sun, and M. Xia, “Purified Anthocyanin Supplementation Reduces Dyslipidemia, Enhances Antioxidant Capacity, and Prevents Insulin Resistance in Diabetic Patients,” *J. Nutr.*, vol. 145, no. 4, pp. 742–748, Apr. 2015.
[380] E. Mah *et al.*, “Cashew consumption reduces total and LDL cholesterol: a randomized, crossover, controlled-feeding trial,” *Am. J. Clin. Nutr.*, vol. 105, no. 5, pp. 1070–1078, May 2017.
[381] P. Ruszkowski, A. Masajtis-Zagajewska, and M. Nowicki, “Effects of combined statin and ACE inhibitor therapy on endothelial function and blood pressure in essential hypertension - a randomised double-blind, placebo controlled crossover study.,” *J. Renin. Angiotensin. Aldosterone. Syst.*, vol. 20, no. 3, p. 1470320319868890, 2019.
[382] M. Bähr, A. Fechner, J. Krämer, M. Kiehntopf, and G. Jahreis, “Lupin protein positively affects plasma LDL cholesterol and LDL:HDL cholesterol ratio in hypercholesterolemic adults after four weeks of supplementation: a randomized, controlled crossover study,” *Nutr. J.*, vol. 12, no. 1, p. 107, Dec. 2013.
[383] J. W. Lampe, “Interindividual differences in response to plant-based diets: implications for cancer risk,” *Am. J. Clin. Nutr.*, vol. 89, no. 5, pp. 1553S-1557S, May 2009.
[384] R. J. Richter, G. P. Jarvik, and C. E. Furlong, “Paraoxonase 1 (PON1) status and substrate hydrolysis,” *Toxicol. Appl. Pharmacol.*, vol. 235, no. 1, pp. 1–9, 2009.
[385] M. Krzystek-Korpacka *et al.*, “Paraoxonase-1 activity in overweight and obese children and adolescents: Association with obesity-related inflammation and oxidative stress,” *Adv. Clin. Exp. Med.*, vol. 22, no. 2, pp. 229–236, 2013.
[386] S. E. Hofer *et al.*, “Association between PON 1 polymorphisms, PON activity and diabetes complications,” *J. Diabetes Complications*, vol. 20, no. 5, pp. 322–328, Oct. 2006.
[387] T. Utarwuthipong, S. Komindr, V. Pakpeankitvatana, S. Songchitsomboon, and T. Thongmuang, “Small dense low-density lipoprotein concentration and oxidative susceptibility changes after consumption of soybean oil, rice bran oil, palm oil and mixed rice bran/palm oil in hypercholesterolaemic women,” *J. Int. Med. Res.*, vol. 37, no. 1, pp. 96–104, 2009.
[388] Y. Hernandez-Diaz *et al.*, “Effects of paraoxonase 1 gene polymorphisms on heart diseases: Systematic review and meta-analysis of 64 case-control studies,” *Med.*, vol. 95, no. 44, p. e5298, 2016.
[389] M. Mackness and B. Mackness, “Human paraoxonase-1 (PON1): Gene structure and expression, promiscuous activities and multiple physiological roles,” *Gene*, vol. 567, no. 1, pp. 12–21, 2015.
[390] M. Castro, “Placebo versus Best-Available-Therapy Control Group in Clinical Trials for Pharmacologic Therapies: Which Is Better?,” *Proc. Am. Thorac. Soc.*, vol. 4, no. 7, p. 570, 2007.
[391] E. J. Mills, A.-W. Chan, P. Wu, A. Vail, G. H. Guyatt, and D. G. Altman, “Design, analysis, and presentation of crossover trials.,” *Trials*, vol. 10, p. 27, Apr. 2009.
[392] J. S. Ratain and M. C. Hochberg, “CLINICAL TRIALS A Guide to Understanding Methodology and Interpreting Results Comparison groups.”
[393] S. Wellek and M. Blettner, “On the proper use of the crossover design in clinical trials: part 18 of a series on evaluation of scientific publications.,” *Dtsch. Arztebl. Int.*, vol. 109, no. 15, pp. 276–81, Apr. 2012.
[394] H. B. Krogh *et al.*, “Methodological advantages and disadvantages of parallel and crossover randomised clinical trials on methylphenidate for attention deficit

hyperactivity disorder: a systematic review and meta-analyses."
[395] I. Krga *et al.*, "Anthocyanins and their gut metabolites reduce the adhesion of monocyte to TNFalpha-activated endothelial cells at physiologically relevant concentrations," *Arch Biochem Biophys*, vol. 599, pp. 51–59, doi:10.1016/j.abb.2016.02.006, Jun. 2016.
[396] L. R. Marques *et al.*, "Reverse Cholesterol Transport: Molecular Mechanisms and the Non-medical Approach to Enhance HDL Cholesterol.," *Front. Physiol.*, vol. 9, p. 526, 2018.
[397] Y. Ji *et al.*, "Promotes High Density Cholesterol Efflux *," *Biochemistry*, vol. 272, no. 10, pp. 20982–20985, 1997.
[398] A. Rohatgi *et al.*, "HDL Cholesterol Efflux Capacity and Incident Cardiovascular Events," *N. Engl. J. Med.*, vol. 371, no. 25, pp. 2383–2393, Dec. 2014.
[399] A. V. Khera and D. J. Rader, "Cholesterol efflux capacity full steam ahead or a bump in the road?," *Arterioscler. Thromb. Vasc. Biol.*, vol. 33, no. 7, pp. 1449–1451, 2013.
[400] E. J. Rhee, C. D. Byrne, and K. C. Sung, "The HDL cholesterol/apolipoprotein A-I ratio: an indicator of cardiovascular disease," *Curr Opin Endocrinol Diabetes Obes*, vol. 24, no. 2, pp. 148–153, 2017.
[401] R. Teimourpour, Z. Meshkat, A. Gholoubi, H. Nomani, and S. Rostami, "Viral load analysis of hepatitis C virus in Huh7.5 cell culture system," *Jundishapur J. Microbiol.*, vol. 8, no. 5, 2015.
[402] B. Sainz, N. Barretto, and S. L. Uprichard, "Hepatitis C virus infection in phenotypically distinct Huh7 cell lines," *PLoS One*, vol. 4, no. 8, 2009.
[403] M. Aviram *et al.*, "Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants," *Free Radic Biol Med*, vol. 26, no. 7–8, pp. 892–904, 1999.
[404] P. Koncsos *et al.*, "Human paraoxonase-1 activity in childhood obesity and its relation to leptin and adiponectin levels," *Pediatr. Res.*, vol. 67, no. 3, pp. 309–313, 2010.
[405] M. P. de Winther, K. W. van Dijk, L. M. Havekes, and M. H. Hofker, "Macrophage scavenger receptor class A: A multifunctional receptor in atherosclerosis," *Arter. Thromb Vasc Biol*, vol. 20, no. 2, pp. 290–297, 2000.
[406] S. P. DEAKIN and R. W. JAMES, "Genetic and environmental factors modulating serum concentrations and activities of the antioxidant enzyme paraoxonase-1," *Clin. Sci.*, vol. 107, no. 5, pp. 435–447, 2004.
[407] L. Badimon and G. Vilahur, "LDL-cholesterol versus HDL-cholesterol in the atherosclerotic plaque: Inflammatory resolution versus thrombotic chaos," *Ann. N. Y. Acad. Sci.*, vol. 1254, no. 1, pp. 18–32, Apr. 2012.

APPENDIX 1

TRANSCRIPTOMIC STUDY:

Regulated pathways and differentially expressed genes in response to anthocyanin metabolites treatment

Table S1. 1: Summary of GSEA using Gene Ontology database for PGA treatment

NAME	ES	NES	FDR
GO_SISTER_CHROMATID_SEGREGATION	0.5498	2.2603	0.0012
GO_MITOTIC_SISTER_CHROMATID_SEGREGATION	0.5959	2.2349	0.0006
GO_NUCLEAR_CHROMOSOME_SEGREGATION	0.5195	2.2084	0.0004
GO_REGULATION_OF_CENTROSOME_CYCLE	0.6585	2.1473	0.0020
GO_CHROMOSOME_SEGREGATION	0.4922	2.1286	0.0023
GO_TRANSLATIONAL_INITIATION	0.5158	2.0956	0.0039
GO_MITOTIC_NUCLEAR_DIVISION	0.4661	2.0952	0.0034
GO_MITOCHONDRIAL_TRANSLATION	0.5299	2.0949	0.0029
GO_SISTER_CHROMATID_COHESION	0.5360	2.0794	0.0034
GO_POSITIVE_REGULATION_OF_COAGULATION	0.7697	2.0792	0.0031
GO_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION_TO_ENDOPLASMIC_RETICULUM	0.5371	2.0586	0.0041
GO_RIBOSOMAL_SMALL_SUBUNIT_BIOGENESIS	0.5882	2.0316	0.0060
GO_RIBOSOME_BIOGENESIS	0.4555	2.0290	0.0058
GO_CELL_CYCLE_PHASE_TRANSITION	0.4641	2.0196	0.0062
GO_TRNA_TRANSPORT	0.6545	2.0128	0.0071
GO_NEGATIVE_REGULATION_OF_AXON_EXTENSION	-0.6732	-1.9822	0.1115
GO_POSITIVE_REGULATION_OF_EXCITATORY_POSTSYNAPTIC_POTENTIAL	-0.7530	-1.9208	0.1608
GO_MODULATION_OF_EXCITATORY_POSTSYNAPTIC_POTENTIAL	-0.6675	-1.8618	0.1934

- GSEA = gene set enrichment analysis, ES = enrichment score, NES = normalized enrichment score, FDR = false discovery rate (adjusted *p*-value)
- Cells highlighted with yellow are significantly changed at FDR ≤ 0.05, while blue cells are significant at FDR ≤ 0.2.
- Positive NES values = upregulated pathways, negative NES values = downregulated pathways

Table S1. 2: Summary of GSEA using Reactome database for PGA treatment

NAME	ES	NES	FDR
INFLUENZA_LIFE_CYCLE	0.553	2.256	0
G1_S_TRANSITION	0.575	2.252	0
CELL_CYCLE_MITOTIC	0.494	2.223	0.001
S_PHASE	0.554	2.155	0.001
MITOTIC_G1_G1_S_PHASES	0.539	2.141	0.001
HOST_INTERACTIONS_OF_HIV_FACTORS	0.537	2.137	0.001
CELL_CYCLE_CHECKPOINTS	0.543	2.137	0.000
DNA_REPLICATION	0.512	2.134	0.000
INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	0.541	2.119	0.001
ORC1_REMOVAL_FROM_CHROMATIN	0.585	2.108	0.001
MITOTIC_M_M_G1_PHASES	0.512	2.100	0.001
METABOLISM_OF_RNA	0.474	2.098	0.000
HIV_INFECTION	0.499	2.081	0.000
CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	0.582	2.076	0.000
REGULATION_OF_MITOTIC_CELL_CYCLE	0.565	2.074	0.000
SMOOTH_MUSCLE_CONTRACTION	-0.584	-1.606	0.299

- GSEA = gene set enrichment analysis, ES = enrichment score, NES = normalized enrichment score, FDR = false discovery rate (adjusted *p*-value).
- Cells highlighted with yellow are significantly changed at FDR ≤ 0.05, while blue cells are significant at FDR ≤ 0.3.
- Positive NES values = upregulated pathways, negative NES values = downregulated pathways

Table S1. 3: Summary of GSEA using Gene Ontology database for mixture treatment

NAME	ES	NES	FDR
RIBOSOMAL_SMALL_SUBUNIT_BIOGENESIS	0.584	2.081	0.046
DNA_DEALKYLATION	0.780	2.040	0.047
MATURATION_OF_SSU_RRNA	0.603	1.961	0.127
MEMBRANE_DISASSEMBLY	0.587	1.950	0.109
REGULATION_OF_CELL_AGING	0.638	1.917	0.141
MATURATION_OF_SSU_RRNA_FROM_TRICISTRONIC_RRNA_TRANSCRIPT_SSU_RRNA_5_8S_RRNA_LSU_RRNA_	0.610	1.908	0.132
ANDROGEN_RECEPTOR_SIGNALING_PATHWAY	0.597	1.901	0.122
REGULATION_OF_CELLULAR_SENESCENCE	0.663	1.873	0.154
REGULATION_OF_CELLULAR_RESPONSE_TO_HEAT	0.521	1.850	0.185
INTRACELLULAR_STEROID_HORMONE_RECEPTOR_SIGNALING_PATHWAY	0.522	1.838	0.186
REGULATION_OF_TRANSCRIPTION_REGULATORY_REGION_DNA_BINDING	0.607	1.827	0.193
SPLICEOSOMAL_SNRNP_ASSEMBLY	0.577	1.818	0.201
REGULATION_OF_SYNAPTIC_VESICLE_TRANSPORT	0.621	1.787	0.271
LONG_TERM_MEMORY	0.642	1.786	0.254
REGULATION_OF_HORMONE_METABOLIC_PROCESS	0.651	1.782	0.250
HUMORAL_IMMUNE_RESPONSE_MEDIATED_BY_CIRCULATING_IMMUNOGLOBULIN	-0.662	-1.927	0.202
ACTIN_MYOSIN_FILAMENT_SLIDING	-0.709	-1.870	0.237

- Mixture treatment is a mixture of PGA, syringic acid and vanillic acid (3.3 μM each).
- GSEA = gene set enrichment analysis, ES = enrichment score, NES = normalized enrichment score, FDR = false discovery rate (adjusted *p*-value).
- Cells highlighted with yellow are significantly changed at FDR ≤ 0.05, while blue cells are significant at FDR ≤ 0.3.
- Positive NES values = upregulated pathways, negative NES values = downregulated pathways

Table S1. 4: Summary of GSEA using Reactome database for mixture treatment.

NAME	ES	NES	FDR
METABOLISM_OF_NON_CODING_RNA	0.592	1.952	0.087
CYTOSOLIC_TRNA_AMINOACYLATION	0.685	1.932	0.060
AMINO_ACID_TRANSPORT_ACROSS_THE_PLASMA_MEMBRANE	0.657	1.810	0.183
GLUCOSE_TRANSPORT	0.581	1.761	0.240
DNA_REPAIR	0.456	1.756	0.201
REGULATION_OF_GLUCOKINASE_BY_GLUCOKINASE_REGULATORY_PROTEIN	0.588	1.729	0.222
TRANSPORT_OF_RIBONUCLEOPROTEINS_INTO_THE_HOST_NUCLEUS	0.582	1.722	0.202
FANCONI_ANEMIA_PATHWAY	0.636	1.718	0.185
TRNA_AMINOACYLATION	0.541	1.703	0.185
RORA_ACTIVATES_CIRCADIAN_EXPRESSION	0.579	1.685	0.195
NEP_NS2_INTERACTS_WITH_THE_CELLULAR_EXPORT_MACHINERY	0.573	1.677	0.189
NOTCH1_INTRACELLULAR_DOMAIN_REGULATES_TRANSCRIPTION	0.492	1.651	0.225
CA_DEPENDENT_EVENTS	0.589	1.647	0.215
INTERACTIONS_OF_VPR_WITH_HOST_CELLULAR_PROTEINS	0.532	1.640	0.211
INFLUENZA_LIFE_CYCLE	0.402	1.639	0.199
INTERFERON_ALPHA_BETA_SIGNALING	-0.530	-1.743	0.288
COMPLEMENT_CASCADE	-0.652	-1.667	0.219
MUSCLE_CONTRACTION	-0.537	-1.622	0.250

- Mixture treatment is a mixture of PGA, syringic acid and vanillic acid (3.3 μM each).
- GSEA = gene set enrichment analysis, ES = enrichment score, NES = normalized enrichment score, FDR = false discovery rate (adjusted *p*-value).
- Cells highlighted with yellow are significantly changed at FDR ≤ 0.05, while blue cells are significant at FDR ≤ 0.3.
- Positive NES values = upregulated pathways, negative NES values = downregulated pathways

Table S1. 5: Summary of GSEA using Gene Ontology database for vanillic acid treatment

NAME	ES	NES	FDR
POSITIVE_REGULATION_OF_VIRAL_GENOME_REPLICATION	0.667	2.031	0.051
CYCLIC_NUCLEOTIDE_CATABOLIC_PROCESS	0.785	2.030	0.026
REGULATION_OF_NUCLEOSIDE_METABOLIC_PROCESS	0.605	1.899	0.161

- GSEA = gene set enrichment analysis, ES = enrichment score, NES = normalized enrichment score, FDR = false discovery rate (adjusted *p*-value).
- Cells highlighted with yellow are significantly changed at FDR ≤ 0.05, while blue cells are significant at FDR ≤ 0.3.
- Positive NES values = upregulated pathways, negative NES values = downregulated pathways

Table S1. 6: Summary of GSEA using Reactome database for vanillic acid treatment.

NAME	ES	NES	FDR
ANTIVIRAL_MECHANISM_BY_IFN_STIMULATED_GENES	0.506	1.756	0.385
TGF_BETA_RECEPTOR_SIGNALING_ACTIVATES_SMADS	-0.672	-1.959	0.043
BASIGIN_INTERACTIONS	-0.637	-1.772	0.325
DOWNREGULATION_OF_TGF_BETA_RECEPTOR_SIGNALING	-0.636	-1.762	0.248
SIGNALING_BY_TGF_BETA_RECEPTOR_COMPLEX	-0.489	-1.715	0.301
SPHINGOLIPID_DE_NOVO_BIOSYNTHESIS	-0.572	-1.662	0.393

- GSEA = gene set enrichment analysis, ES = enrichment score, NES = normalized enrichment score, FDR = false discovery rate (adjusted *p*-value).
- Cells highlighted with yellow are significantly changed at FDR ≤ 0.05, while blue cells are significant at FDR ≤ 0.4.
- Positive NES values = upregulated pathways, negative NES values = downregulated pathways.

Table S1. 7: Summary of GSEA using Gene Ontology database for syringic acid treatment

NAME	ES	NES	FDR
CAMP_METABOLIC_PROCESS	0.737	2.070	0.034
AORTA_DEVELOPMENT	0.664	2.066	0.018
CORONARY_VASCULATURE_DEVELOPMENT	0.673	2.046	0.016
POSITIVE_REGULATION_OF_TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER_INVOLVED_IN_CELLULAR_RESPONSE_TO_CHEMICAL_STIMULUS	0.726	1.993	0.032
EMBRYONIC_CAMERA_TYPE_EYE_DEVELOPMENT	0.706	1.976	0.034
CYCLIC_NUCLEOTIDE_METABOLIC_PROCESS	0.625	1.959	0.038
CYCLIC_NUCLEOTIDE_CATABOLIC_PROCESS	0.766	1.953	0.036
RESPONSE_TO_ACTIVITY	0.602	1.936	0.042
EMBRYONIC_EYE_MORPHOGENESIS	0.695	1.922	0.047
ARTERY_DEVELOPMENT	0.553	1.894	0.066
AORTA_MORPHOGENESIS	0.682	1.856	0.106
POSITIVE_REGULATION_OF_FAT_CELL_DIFFERENTIATION	0.604	1.855	0.099
REGULATION_OF_PROTEIN_IMPORT_INTO_NUCLEUS_TRANSLOCATION	0.692	1.835	0.116
REGULATION_OF_SYNAPTIC_TRANSMISSION_GABAERGIC	0.683	1.816	0.140
EYE_MORPHOGENESIS	0.500	1.807	0.146
DNA_STRAND_ELONGATION_INVOLVED_IN_DNA_REPLICATION	-0.674	-1.952	0.203
DNA_STRAND_ELONGATION	-0.672	-1.936	0.130
NUCLEOTIDE_EXCISION_REPAIR_DNA_GAP_FILLING	-0.634	-1.824	0.285

- GSEA = gene set enrichment analysis, ES = enrichment score, NES = normalized enrichment score, FDR = false discovery rate (adjusted *p*-value).
- Cells highlighted with yellow are significantly changed at FDR ≤ 0.05, while blue cells are significant at FDR ≤ 0.3.
- Positive NES values = upregulated pathways, negative NES values = downregulated pathways.

Table S1. 8: Summary of GSEA using Reactom database for syringic acid treatment

NAME	ES	NES	FDR
RORA_ACTIVATES_CIRCADIAN_EXPRESSION	0.669	1.886	0.110
CIRCADIAN_REPRESSION_OF_EXPRESSION_BY_REV_ERBA	0.675	1.875	0.068
GLOBAL_GENOMIC_NER_GG_NER	-0.629	-1.992	0.029
S_PHASE	-0.502	-1.976	0.019
SYNTHESIS_OF_DNA	-0.498	-1.874	0.046
DNA_STRAND_ELONGATION	-0.613	-1.874	0.035
LAGGING_STRAND_SYNTHESIS	-0.702	-1.852	0.038
M_G1_TRANSITION	-0.493	-1.810	0.051
G1_S_TRANSITION	-0.458	-1.778	0.064
ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	-0.559	-1.765	0.063
ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	-0.564	-1.759	0.059
EXTENSION_OF_TELOMERES	-0.585	-1.705	0.094
DNA_REPLICATION	-0.408	-1.695	0.095
TELOMERE_MAINTENANCE	-0.493	-1.692	0.089
FORMATION_OF_INCISION_COMPLEX_IN_GG_NER	-0.582	-1.638	0.131
CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	-0.458	-1.628	0.133
MITOTIC_M_M_G1_PHASES	-0.392	-1.624	0.129

- GSEA = gene set enrichment analysis, ES = enrichment score, NES = normalized enrichment score, FDR = false discovery rate (adjusted *p*-value).
- Cells highlighted with yellow are significantly changed at FDR ≤ 0.05, while blue cells are significant at FDR ≤ 0.25.
- Positive NES values = upregulated pathways, negative NES values = downregulated pathways.

Table S1. 9: list of upregulated genes by phloroglucinaldehyde (10 µM) in THP-1 macrophages at adjusted p-value ≤ 0.3 using RNA sequencing

Gene name	Ensembl ID	Log fold change	*p*-value	Adjusted *p*-value	Fold change
RPGRIP1L	ENSG00000103494	0.51	0.00	0.11	1.4
CD36	ENSG00000135218	0.27	0.00	0.13	1.2
NA	NA	0.54	0.00	0.14	1.4
FABP4	ENSG00000170323	0.40	0.00	0.14	1.3
HACL1	ENSG00000131373	0.28	0.00	0.14	1.2
MAT2B	ENSG00000038274	0.23	0.00	0.14	1.2
FABP5	ENSG00000164687	0.19	0.00	0.14	1.1
RBL1	ENSG00000080839	0.21	0.00	0.14	1.2
PLIN2	ENSG00000147872	0.18	0.00	0.16	1.1
SSB	ENSG00000138385	0.21	0.00	0.17	1.2
MFAP1	ENSG00000140259	0.23	0.00	0.19	1.2
WDR48	ENSG00000114742	0.22	0.00	0.20	1.2
DHX9	ENSG00000135829	0.19	0.00	0.21	1.1
REPS1	ENSG00000135597	0.20	0.00	0.21	1.1
ASXL2	ENSG00000143970	1.25	0.00	0.22	2.4
NA	NA	0.64	0.00	0.22	1.6
PSAT1	ENSG00000135069	0.24	0.00	0.24	1.2
OPN3	ENSG00000054277	0.21	0.00	0.24	1.2
SEC24B	ENSG00000138802	0.21	0.00	0.24	1.2
HHLA3	ENSG00000197568	0.76	0.00	0.25	1.7
CCNG2	ENSG00000138764	0.52	0.00	0.25	1.4
WTH3DI	ENSG00000233087	0.52	0.00	0.25	1.4
NA	NA	0.51	0.00	0.25	1.4
C5orf42	ENSG00000197603	0.35	0.00	0.25	1.3
NA	NA	0.29	0.00	0.25	1.2
GBP3	ENSG00000117226	0.29	0.00	0.25	1.2
PLA2G12A	ENSG00000123739	0.27	0.00	0.25	1.2
LRRC4	ENSG00000128594	2.26	0.00	0.25	4.8
AC006111.2	ENSG00000263235	0.17	0.00	0.25	1.1
NA	NA	0.52	0.00	0.26	1.4
AC009303.4	ENSG00000279227	0.90	0.00	0.26	1.9
NACA2	ENSG00000253506	0.72	0.00	0.26	1.7
GPT2	ENSG00000166123	0.52	0.00	0.26	1.4
ASIC1	ENSG00000110881	0.50	0.00	0.26	1.4
PLEKHG1	ENSG00000120278	0.46	0.00	0.26	1.4
SKA3	ENSG00000165480	0.42	0.00	0.26	1.3
NFYA	ENSG00000001167	0.41	0.00	0.26	1.3
NPAT	ENSG00000149308	0.35	0.00	0.26	1.3
CAAP1	ENSG00000120159	0.30	0.00	0.26	1.2
C4orf3	ENSG00000164096	0.29	0.00	0.26	1.2

Table S1.9 continued

Gene name	Ensembl ID	Log fold change	*p*-value	Adjusted *p*-value	Fold change
CFAP36	ENSG00000163001	0.28	0.00	0.26	1.2
LINC00909	ENSG00000264247	0.28	0.00	0.26	1.2
C5orf34	ENSG00000172244	0.26	0.00	0.26	1.2
PIBF1	ENSG00000083535	0.25	0.00	0.26	1.2
SPG20	ENSG00000133104	0.24	0.00	0.26	1.2
TMEM55A	ENSG00000155099	0.22	0.00	0.26	1.2
DYNC1I2	ENSG00000077380	0.22	0.00	0.26	1.2
RPS3AP6	ENSG00000234797	0.21	0.00	0.26	1.2
MRPS30	ENSG00000112996	0.20	0.00	0.26	1.2
ERMP1	ENSG00000099219	0.20	0.00	0.26	1.1
ADCY1	ENSG00000164742	0.19	0.00	0.26	1.1
SCOC	ENSG00000153130	0.19	0.00	0.26	1.1
TLR4	ENSG00000136869	0.19	0.00	0.26	1.1
HIF1A	ENSG00000100644	0.15	0.00	0.26	1.1
SLC30A5	ENSG00000145740	0.15	0.00	0.26	1.1
RCN2	ENSG00000117906	0.15	0.00	0.26	1.1
PPIH	ENSG00000171960	0.13	0.00	0.26	1.1
HMGB1P6	ENSG00000259781	0.13	0.00	0.26	1.1
MIR5703	ENSG00000284092	0.13	0.00	0.26	1.1
FEM1B	ENSG00000169018	0.12	0.00	0.26	1.1
PEBP1	ENSG00000089220	0.11	0.00	0.26	1.1
PLPBP	ENSG00000147471	0.11	0.00	0.26	1.1
RPL29P11	ENSG00000224858	0.34	0.00	0.26	1.3
HSPE1	ENSG00000115541	0.49	0.00	0.28	1.4
HAT1	ENSG00000128708	0.15	0.00	0.28	1.1
RPSAP12	ENSG00000240087	0.32	0.00	0.28	1.2
MRPL46	ENSG00000259494	0.28	0.00	0.28	1.2
ENPP4	ENSG00000001561	0.20	0.00	0.28	1.2
NCEH1	ENSG00000144959	0.19	0.00	0.28	1.1
SMARCA5	ENSG00000153147	0.16	0.00	0.28	1.1
CYCS	ENSG00000172115	0.14	0.00	0.28	1.1
DCLK3	ENSG00000163673	0.42	0.00	0.28	1.3
NEK1	ENSG00000137601	0.29	0.00	0.28	1.2
DDX10	ENSG00000178105	0.26	0.00	0.28	1.2
AC009487.2	ENSG00000226121	0.19	0.00	0.28	1.1
RALGAPB	ENSG00000170471	0.18	0.00	0.28	1.1
CCT4	ENSG00000115484	0.20	0.00	0.28	1.1
MTMR9	ENSG00000104643	0.15	0.00	0.28	1.1
GFM1	ENSG00000168827	0.14	0.00	0.28	1.1
SCD	ENSG00000099194	0.17	0.00	0.28	1.1

Table S1.9 continued

Gene name	Ensembl ID	Log fold change	*p*-value	Adjusted *p*-value	Fold change
SACS	ENSG00000151835	0.29	0.00	0.29	1.2
GBE1	ENSG00000114480	0.17	0.00	0.29	1.1
PCM1	ENSG00000078674	0.11	0.00	0.29	1.1
USP28	ENSG00000048028	0.26	0.00	0.29	1.2
ERCC8	ENSG00000049167	0.25	0.00	0.29	1.2
PM20D2	ENSG00000146281	0.23	0.00	0.29	1.2
SNRPD1	ENSG00000167088	0.19	0.00	0.29	1.1
ABL2	ENSG00000143322	0.23	0.00	0.29	1.2
TMSB10	ENSG00000034510	0.10	0.00	0.29	1.1
ERCC6L	ENSG00000186871	0.29	0.00	0.29	1.2
PABPC1	ENSG00000070756	0.21	0.00	0.29	1.2
NA	NA	1.30	0.01	0.30	2.5
LINC00942	ENSG00000249628	1.21	0.01	0.30	2.3
MAGEA4	ENSG00000147381	1.02	0.01	0.30	2.0
ZNF35	ENSG00000169981	0.99	0.01	0.30	2.0
NA	NA	0.93	0.01	0.30	1.9
AC116347.1	ENSG00000238000	0.87	0.01	0.30	1.8
CDKL1	ENSG00000100490	0.75	0.01	0.30	1.7
DMC1	ENSG00000100206	0.63	0.01	0.30	1.6
WDR31	ENSG00000148225	0.57	0.00	0.30	1.5
AL590764.1	ENSG00000228427	0.52	0.01	0.30	1.4
AC004080.2	ENSG00000253508	0.46	0.01	0.30	1.4
BCYRN1	ENSG00000236824	0.42	0.00	0.30	1.3
PLOD2	ENSG00000152952	0.41	0.01	0.30	1.3
ZNF830	ENSG00000198783	0.36	0.00	0.30	1.3
FKBP7	ENSG00000079150	0.35	0.01	0.30	1.3
TTC21B	ENSG00000123607	0.31	0.01	0.30	1.2
SPIN4	ENSG00000186767	0.30	0.00	0.30	1.2
EXTL2	ENSG00000162694	0.29	0.00	0.30	1.2
CALN1	ENSG00000183166	0.28	0.00	0.30	1.2
TOGARAM1	ENSG00000198718	0.28	0.01	0.30	1.2
NA	NA	0.27	0.01	0.30	1.2
TPM3P9	ENSG00000241015	0.26	0.01	0.30	1.2
CARNMT1	ENSG00000156017	0.25	0.01	0.30	1.2
AKAP11	ENSG00000023516	0.23	0.01	0.30	1.2
RPL36	ENSG00000130255	0.22	0.00	0.30	1.2
UBE2C	ENSG00000175063	0.22	0.01	0.30	1.2
AC091959.3	ENSG00000275740	0.22	0.01	0.30	1.2
TXNL4B	ENSG00000140830	0.21	0.01	0.30	1.2
MRPL39	ENSG00000154719	0.21	0.01	0.30	1.2

Table S1.9 continued

Gene name	Ensembl ID	Log fold change	*p*-value	Adjusted *p*-value	Fold change
STAG1	ENSG00000118007	0.20	0.01	0.30	1.2
ZKSCAN7	ENSG00000196345	0.20	0.01	0.30	1.1
MRPL1	ENSG00000169288	0.19	0.01	0.30	1.1
SECISBP2L	ENSG00000138593	0.19	0.01	0.30	1.1
SUZ12	ENSG00000178691	0.18	0.00	0.30	1.1
ARRDC3	ENSG00000113369	0.18	0.01	0.30	1.1
UBA6	ENSG00000033178	0.17	0.00	0.30	1.1
LANCL1	ENSG00000115365	0.17	0.01	0.30	1.1
ACAP2	ENSG00000114331	0.16	0.01	0.30	1.1
RFC1	ENSG00000035928	0.15	0.01	0.30	1.1
SESN3	ENSG00000149212	0.15	0.01	0.30	1.1
AGPS	ENSG00000018510	0.15	0.01	0.30	1.1
TMSB4XP8	ENSG00000187653	0.15	0.00	0.30	1.1
SRPK1	ENSG00000096063	0.15	0.01	0.30	1.1
CDK1	ENSG00000170312	0.14	0.01	0.30	1.1
PDS5A	ENSG00000121892	0.14	0.01	0.30	1.1
RCAN1	ENSG00000159200	0.14	0.00	0.30	1.1
RUNDC1	ENSG00000198863	0.14	0.01	0.30	1.1
TMA7	ENSG00000232112	0.13	0.01	0.30	1.1
IRF2	ENSG00000168310	0.13	0.00	0.30	1.1
PJA2	ENSG00000198961	0.11	0.01	0.30	1.1
EIF1AX	ENSG00000173674	0.11	0.01	0.30	1.1
ZMPSTE24	ENSG00000084073	0.10	0.01	0.30	1.1
TOP1	ENSG00000198900	0.10	0.01	0.30	1.1
PLEK	ENSG00000115956	0.10	0.01	0.30	1.1
HNRNPA1P7	ENSG00000215492	0.12	0.01	0.30	1.1

Table S1. 10: list of downregulated genes by phloroglucinaldehyde (10 µM) in THP-1 macrophages at adjusted p-value ≤ 0.3 using RNA sequencing

Gene name	EnsemblID	Log fold change	*p*-Value	Adjusted *p*-value	Fold change
ZBTB14	ENSG00000198081	-0.87	0.00	0.00	0.55
RN7SL5P	ENSG00000265735	-2.11	0.00	0.05	0.23
AC133065.2	ENSG00000262222	-1.26	0.00	0.09	0.42
MED31	ENSG00000108590	-0.47	0.00	0.09	0.72
HSPA7	ENSG00000225217	-1.42	0.00	0.11	0.37
SEMA6A	ENSG00000092421	-0.69	0.00	0.11	0.62
UNC93B1	ENSG00000110057	-0.23	0.00	0.11	0.85
RN7SL4P	ENSG00000263740	-2.16	0.00	0.13	0.22
RN7SL2	ENSG00000274012	-1.92	0.00	0.13	0.26
NA	NA	-6.97	0.00	0.14	0.01
AC092384.2	ENSG00000259881	-1.50	0.00	0.14	0.35
SNHG17	ENSG00000196756	-0.42	0.00	0.14	0.75
IDUA	ENSG00000127415	-0.41	0.00	0.14	0.75
CCDC88B	ENSG00000168071	-0.32	0.00	0.14	0.80
7SK	ENSG00000202198	-2.88	0.00	0.15	0.14
C7	ENSG00000112936	-5.18	0.00	0.16	0.03
SP140L	ENSG00000185404	-0.57	0.00	0.17	0.67
PIDD1	ENSG00000177595	-0.38	0.00	0.17	0.77
MIB2	ENSG00000197530	-0.32	0.00	0.17	0.80
OPLAH	ENSG00000178814	-0.26	0.00	0.19	0.83
CD37	ENSG00000104894	-0.26	0.00	0.19	0.84
ERP27	ENSG00000139055	-0.52	0.00	0.20	0.70
RN7SKP203	ENSG00000200488	-2.71	0.00	0.20	0.15
IRF5	ENSG00000128604	-0.41	0.00	0.21	0.75
AL139099.5	ENSG00000283029	-1.43	0.00	0.21	0.37
NLGN2	ENSG00000169992	-0.81	0.00	0.21	0.57
SYTL1	ENSG00000142765	-0.54	0.00	0.21	0.69
ARL2	ENSG00000213465	-0.41	0.00	0.21	0.75
ADGRL1	ENSG00000072071	-0.37	0.00	0.21	0.77
RELT	ENSG00000054967	-0.17	0.00	0.22	0.89
PHLDB2	ENSG00000144824	-3.84	0.00	0.23	0.07
PDXDC2P-NPIPB14P	ENSG00000196696	-0.50	0.00	0.23	0.70
KCNQ1OT1	ENSG00000269821	-0.45	0.00	0.24	0.73
NA	NA	-3.88	0.00	0.24	0.07
CHKA	ENSG00000110721	-0.29	0.00	0.24	0.82
UMOD	ENSG00000169344	-3.76	0.00	0.25	0.07
YTHDF2P1	ENSG00000270503	-3.13	0.00	0.25	0.11
UBE2Q2P1	ENSG00000189136	-0.97	0.00	0.25	0.51

Table S1.10 continued

Gene name	EnsemblID	Log fold change	*p*-Value	Adjusted *p*-value	Fold change
MZF1	ENSG00000099326	-0.53	0.00	0.25	0.69
NA	NA	-0.47	0.00	0.25	0.72
ANKS3	ENSG00000168096	-0.45	0.00	0.25	0.73
RHBDF1	ENSG00000007384	-0.37	0.00	0.25	0.77
IRF7	ENSG00000185507	-0.36	0.00	0.25	0.78
PARVG	ENSG00000138964	-0.26	0.00	0.25	0.84
PKN3	ENSG00000160447	-0.23	0.00	0.25	0.85
PNPLA2	ENSG00000177666	-0.22	0.00	0.25	0.86
ARHGEF1	ENSG00000076928	-0.22	0.00	0.25	0.86
NAPRT	ENSG00000147813	-0.15	0.00	0.25	0.90
PNKD	ENSG00000127838	-0.14	0.00	0.25	0.91
AC233280.2	ENSG00000271662	-1.41	0.00	0.25	0.38
MST1L	ENSG00000186715	-0.73	0.00	0.25	0.60
NOS2	ENSG00000007171	-0.40	0.00	0.25	0.76
ALOX5	ENSG00000012779	-0.26	0.00	0.25	0.83
RN7SL3	ENSG00000278771	-1.34	0.00	0.25	0.39
NA	NA	-1.04	0.00	0.25	0.49
IKBKGP1	ENSG00000275882	-0.19	0.00	0.26	0.88
ALDOB	ENSG00000136872	-3.10	0.00	0.26	0.12
ABHD17AP4	ENSG00000229107	-3.09	0.00	0.26	0.12
LIMCH1	ENSG00000064042	-2.94	0.00	0.26	0.13
RMRP	ENSG00000269900	-1.89	0.00	0.26	0.27
ADAMTS12	ENSG00000151388	-1.80	0.00	0.26	0.29
TSACC	ENSG00000163467	-1.41	0.00	0.26	0.38
CUBN	ENSG00000107611	-1.37	0.00	0.26	0.39
C11orf94	ENSG00000234776	-1.30	0.00	0.26	0.41
SNORD3A	ENSG00000263934	-1.19	0.00	0.26	0.44
AL589935.1	ENSG00000232295	-1.09	0.00	0.26	0.47
AC006277.1	ENSG00000279452	-0.94	0.00	0.26	0.52
NA	NA	-0.91	0.00	0.26	0.53
MEPE	ENSG00000152595	-0.90	0.00	0.26	0.54
TAS1R3	ENSG00000169962	-0.86	0.00	0.26	0.55
LRRC24	ENSG00000254402	-0.81	0.00	0.26	0.57
MIR503HG	ENSG00000223749	-0.78	0.00	0.26	0.58
LBX2	ENSG00000179528	-0.64	0.00	0.26	0.64
ANO9	ENSG00000185101	-0.63	0.00	0.26	0.65
CACTIN-AS1	ENSG00000226800	-0.57	0.00	0.26	0.67
LIMS2	ENSG00000072163	-0.53	0.00	0.26	0.69

Table S1.10 continued

Gene name	EnsemblID	Log fold change	*p*-Value	Adjusted *p*-value	Fold change
AC080038.1	ENSG00000274565	-0.53	0.00	0.26	0.69
OSMR	ENSG00000145623	-0.46	0.00	0.26	0.73
AC087499.1	ENSG00000188013	-0.42	0.00	0.26	0.75
CENPT	ENSG00000102901	-0.41	0.00	0.26	0.75
TRPT1	ENSG00000149743	-0.40	0.00	0.26	0.76
SLC22A31	ENSG00000259803	-0.39	0.00	0.26	0.77
SLC22A18	ENSG00000110628	-0.36	0.00	0.26	0.78
WDR24	ENSG00000127580	-0.35	0.00	0.26	0.78
NEK8	ENSG00000160602	-0.34	0.00	0.26	0.79
IFFO1	ENSG00000010295	-0.33	0.00	0.26	0.80
KIAA1614	ENSG00000135835	-0.33	0.00	0.26	0.80
ADCK5	ENSG00000173137	-0.31	0.00	0.26	0.81
SUSD3	ENSG00000157303	-0.31	0.00	0.26	0.81
DENND6B	ENSG00000205593	-0.31	0.00	0.26	0.81
ABCA7	ENSG00000064687	-0.30	0.00	0.26	0.81
LPAR2	ENSG00000064547	-0.30	0.00	0.26	0.81
FCHO1	ENSG00000130475	-0.26	0.00	0.26	0.83
CECR6	ENSG00000183307	-0.26	0.00	0.26	0.84
POLR2J4	ENSG00000214783	-0.26	0.00	0.26	0.84
ARRDC2	ENSG00000105643	-0.23	0.00	0.26	0.85
WDR55	ENSG00000120314	-0.21	0.00	0.26	0.86
UBA7	ENSG00000182179	-0.21	0.00	0.26	0.87
FASTK	ENSG00000164896	-0.20	0.00	0.26	0.87
DGAT1	ENSG00000185000	-0.20	0.00	0.26	0.87
PPP1R16A	ENSG00000160972	-0.19	0.00	0.26	0.88
ARRDC1	ENSG00000197070	-0.18	0.00	0.26	0.88
TMEM55B	ENSG00000165782	-0.18	0.00	0.26	0.88
TMEM259	ENSG00000182087	-0.18	0.00	0.26	0.88
JAG2	ENSG00000184916	-0.17	0.00	0.26	0.89
CHI3L1	ENSG00000133048	-0.15	0.00	0.26	0.90
NDUFS7	ENSG00000115286	-0.15	0.00	0.26	0.90
PPP1R12C	ENSG00000125503	-0.14	0.00	0.26	0.90
ARSA	ENSG00000100299	-0.14	0.00	0.26	0.91
P3H1	ENSG00000117385	-0.13	0.00	0.26	0.92
SCRIB	ENSG00000180900	-0.11	0.00	0.26	0.92
SLC12A9	ENSG00000146828	-0.23	0.00	0.27	0.85
NA	NA	-1.82	0.00	0.28	0.28
AC051649.2	ENSG00000283787	-0.83	0.00	0.28	0.56
GPC2	ENSG00000213420	-0.71	0.00	0.28	0.61

Table S1.10 continued

Gene name	EnsemblID	Log fold change	*p*-Value	Adjusted *p*-value	Fold change
AC024270.1	ENSG00000166503	-0.47	0.00	0.28	0.72
IRF3	ENSG00000126456	-0.39	0.00	0.28	0.76
OFD1	ENSG00000046651	-0.17	0.00	0.28	0.89
NA	NA	-2.33	0.00	0.28	0.20
SYNE2	ENSG00000054654	-1.76	0.00	0.28	0.30
AP001059.2	ENSG00000275799	-0.94	0.00	0.28	0.52
AL365277.1	ENSG00000182109	-0.45	0.00	0.28	0.73
PTPRM	ENSG00000173482	-2.43	0.00	0.28	0.19
AC138356.1	ENSG00000183154	-0.54	0.00	0.28	0.69
IFNAR2	ENSG00000159110	-0.53	0.00	0.28	0.69
SSPO	ENSG00000197558	-0.48	0.00	0.28	0.72
MST1P2	ENSG00000186301	-0.39	0.00	0.28	0.76
DOK3	ENSG00000146094	-0.20	0.00	0.28	0.87
GSDMD	ENSG00000104518	-0.19	0.00	0.28	0.88
TGFB1	ENSG00000105329	-0.15	0.00	0.28	0.90
AC007952.4	ENSG00000262202	-2.06	0.00	0.28	0.24
AC096733.2	ENSG00000273472	-0.70	0.00	0.28	0.62
CCDC130	ENSG00000104957	-0.43	0.00	0.28	0.74
ZNF785	ENSG00000197162	-0.41	0.00	0.28	0.76
NA	NA	-0.40	0.00	0.28	0.76
GRAMD4	ENSG00000075240	-0.13	0.00	0.28	0.92
BAIAP2L2	ENSG00000128298	-1.80	0.00	0.28	0.29
GDPD5	ENSG00000158555	-0.19	0.00	0.29	0.88
AC127521.1	ENSG00000262823	-1.11	0.00	0.29	0.46
PDLIM7	ENSG00000196923	-0.21	0.00	0.29	0.86
SAMD10	ENSG00000130590	-1.21	0.00	0.29	0.43
MADCAM1	ENSG00000099866	-1.17	0.00	0.29	0.45
SLC11A1	ENSG00000018280	-0.31	0.00	0.29	0.81
NADK	ENSG00000008130	-0.20	0.00	0.29	0.87
RBBP4	ENSG00000162521	-0.44	0.00	0.29	0.74
PGA4	ENSG00000229183	-0.40	0.00	0.29	0.76
PNPLA7	ENSG00000130653	-0.35	0.00	0.29	0.79
ASB16-AS1	ENSG00000267080	-0.31	0.00	0.29	0.81
AL031846.2	ENSG00000279833	-0.85	0.00	0.29	0.55
TCAF2	ENSG00000170379	-0.71	0.00	0.29	0.61
MBD6	ENSG00000166987	-0.35	0.00	0.29	0.78
RAB44	ENSG00000255587	-0.29	0.00	0.29	0.82
AC090114.2	ENSG00000273270	-0.26	0.00	0.29	0.84
DHTKD1	ENSG00000181192	-0.20	0.00	0.29	0.87
AP5Z1	ENSG00000242802	-0.18	0.00	0.29	0.88

Table S1.10 continued

Gene name	EnsemblID	Log fold change	*p*-Value	Adjusted *p*-value	Fold change
OGFR	ENSG00000060491	-0.13	0.00	0.29	0.91
CYBA	ENSG00000051523	-0.11	0.00	0.29	0.92
AC048382.5	ENSG00000275120	-2.74	0.00	0.29	0.15
SDCBP2	ENSG00000125775	-0.77	0.00	0.29	0.59
COL9A2	ENSG00000049089	-0.35	0.00	0.29	0.78
ARHGAP27P1-BPTFP1-KPNA2P3	ENSG00000215769	-0.33	0.00	0.29	0.80
MAN2C1	ENSG00000140400	-0.30	0.00	0.29	0.81
ZMIZ2	ENSG00000122515	-0.21	0.00	0.29	0.86
STK25	ENSG00000115694	-0.18	0.00	0.29	0.89
ELAC1	ENSG00000141642	-0.17	0.00	0.29	0.89
NBDY	ENSG00000204272	-0.72	0.00	0.29	0.61
AQP2	ENSG00000167580	-3.46	0.01	0.30	0.09
PCK1	ENSG00000124253	-2.53	0.01	0.30	0.17
AC021016.3	ENSG00000273361	-2.24	0.00	0.30	0.21
PLP1	ENSG00000123560	-1.95	0.01	0.30	0.26
AL020996.1	ENSG00000228172	-1.07	0.00	0.30	0.48
TMEM79	ENSG00000163472	-1.01	0.01	0.30	0.50
AC138028.6	ENSG00000278341	-0.94	0.01	0.30	0.52
EPS8L2	ENSG00000177106	-0.87	0.01	0.30	0.55
OLFM3	ENSG00000118733	-0.78	0.01	0.30	0.58
DNM1P35	ENSG00000246877	-0.71	0.00	0.30	0.61
PROC	ENSG00000115718	-0.69	0.01	0.30	0.62
AL355075.5	ENSG00000259162	-0.67	0.01	0.30	0.63
AC073957.3	ENSG00000273151	-0.66	0.01	0.30	0.63
AC005253.1	ENSG00000268030	-0.66	0.00	0.30	0.63
AC012615.6	ENSG00000267244	-0.65	0.01	0.30	0.64
NA	NA	-0.61	0.00	0.30	0.66
MLLT11	ENSG00000213190	-0.59	0.01	0.30	0.66
AL390066.1	ENSG00000224950	-0.59	0.00	0.30	0.67
RPARP-AS1	ENSG00000269609	-0.54	0.01	0.30	0.69
ZNF789	ENSG00000198556	-0.52	0.01	0.30	0.70
MROH6	ENSG00000204839	-0.50	0.01	0.30	0.71
ZFP41	ENSG00000181638	-0.49	0.00	0.30	0.71
CEACAM4	ENSG00000105352	-0.46	0.01	0.30	0.73
ATG16L2	ENSG00000168010	-0.44	0.01	0.30	0.73
AMT	ENSG00000145020	-0.44	0.00	0.30	0.74
CAPRIN2	ENSG00000110888	-0.42	0.01	0.30	0.75
AKT2	ENSG00000105221	-0.41	0.01	0.30	0.76
ADAMTS13	ENSG00000160323	-0.40	0.01	0.30	0.76

Table S1.10 continued

Gene name	EnsemblID	Log fold change	*p*-Value	Adjusted *p*-value	Fold change
MTMR9LP	ENSG00000220785	-0.40	0.00	0.30	0.76
NPIPB12	ENSG00000169203	-0.39	0.01	0.30	0.76
AC244502.1	ENSG00000251002	-0.39	0.01	0.30	0.76
STOM	ENSG00000148175	-0.37	0.00	0.30	0.77
ZNF783	ENSG00000204946	-0.34	0.01	0.30	0.79
IL3RA	ENSG00000185291	-0.33	0.00	0.30	0.80
SUGP2	ENSG00000064607	-0.33	0.01	0.30	0.80
STAG3L5P	ENSG00000242294	-0.33	0.01	0.30	0.80
DCAF8	ENSG00000132716	-0.30	0.01	0.30	0.81
SUZ12P1	ENSG00000264538	-0.29	0.01	0.30	0.82
ADCY4	ENSG00000129467	-0.29	0.01	0.30	0.82
ZNF586	ENSG00000083828	-0.28	0.00	0.30	0.82
DYNLL2	ENSG00000264364	-0.27	0.01	0.30	0.83
TMEM80	ENSG00000177042	-0.27	0.01	0.30	0.83
MMP17	ENSG00000198598	-0.26	0.01	0.30	0.83
ZNF511	ENSG00000198546	-0.26	0.01	0.30	0.83
PRR14	ENSG00000156858	-0.25	0.01	0.30	0.84
IL21R	ENSG00000103522	-0.25	0.01	0.30	0.84
MYO1G	ENSG00000136286	-0.24	0.01	0.30	0.85
CBS	ENSG00000160200	-0.24	0.00	0.30	0.85
MICAL1	ENSG00000135596	-0.22	0.01	0.30	0.86
GBA2	ENSG00000070610	-0.21	0.01	0.30	0.87
MRI1	ENSG00000037757	-0.17	0.00	0.30	0.89
PRKAB1	ENSG00000111725	-0.16	0.01	0.30	0.89
PITPNM1	ENSG00000110697	-0.16	0.01	0.30	0.90
L1CAM	ENSG00000198910	-0.15	0.00	0.30	0.90
REXO1	ENSG00000079313	-0.14	0.00	0.30	0.91
ABCD1	ENSG00000101986	-0.13	0.01	0.30	0.91
NCF4	ENSG00000100365	-0.12	0.01	0.30	0.92
NECTIN2	ENSG00000130202	-0.12	0.00	0.30	0.92
ICAM5	ENSG00000105376	-0.44	0.01	0.30	0.74
FSCN1	ENSG00000075618	-0.08	0.01	0.30	0.95
TPM2	ENSG00000198467	-0.46	0.01	0.30	0.73
STX1A	ENSG00000106089	-0.42	0.01	0.30	0.75
KIFC2	ENSG00000167702	-0.52	0.01	0.30	0.70
DNPEP	ENSG00000123992	-0.16	0.01	0.30	0.89
NA	NA	-0.59	0.01	0.30	0.67

APPENDIX 2

BERI STUDY PROTOCOL

The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults

Short Title: BERI study

STUDY PROTOCOL

Contents

1. Summary

Human intervention trials with anthocyanin-rich berry fruits/extracts such as whole strawberries, elderberry juice and whortleberry extracts, and purified anthocyanins from a mixture of blackcurrant and bilberry have been shown to beneficially alter biological markers for CVD risk, with beneficial effects on lipid profiles common across these studies. The primary aim of the current study is to directly compare the two major types of anthocyanins found in the diet; cyanidin-type and delphinidin-type. In a double-blind, randomized, 3-arm crossover trial we aim to recruit 50 participants to consume capsules containing (i) a bilberry extract providing 320 mg anthocyanins (delphinidin-type), (ii) a black rice extract providing 320 mg anthocyanins (cyanidin-type) and (iii) a placebo control. Capsules for each treatment arm will be consumed for a period of 28 days with a washout period of at least 4 weeks in-between. Samples will be collected at the start (day 1) and end (day 29) of each treatment period. Changes in circulating LDL-Cholesterol will be evaluated as a primary outcome measure in this study. In addition, total/HDL_cholesterol & triglycerides, other markers for cardio-metabolic risk (blood glucose, fructosamine and insulin), markers for lipid metabolism (cholesterol efflux capacity & PON-1 activity) and circulating bile acids and bile acid derivatives will be evaluated. In a subset of participants (n= 24) we will also investigate the relationship between bilberry and black rice anthocyanin ingestion on RNA expression profiles that are known to be involved in atherosclerosis, non-alcoholic fatty liver disease and reverse cholesterol transport as well as faecal excretion of bile acids and lipids. Should more resources become available we will also determine the effects of the interventions on cardio-metabolic disease related microRNA's and gut microbiota profiles in this subset of participants. Dr Paul Kroon (a Quadram Institute Bioscience Research Leader) is Chief Investigator for this study. The study will be conducted and managed at the Quadram Institute Bioscience (QIB) by Wendy Hollands (research scientist/study manager). Other members of the research group (Dr Priscilla Day, Dr Natalia Perez, Mark Philo, Jasmine Percival, Emad Shehata, Dr Supriya Yadav and Hassan Aboufarrag) will be required to assist. This study is funded through the Food and Health Institute Strategic Programme grant to QIB.

2. Scientific Background

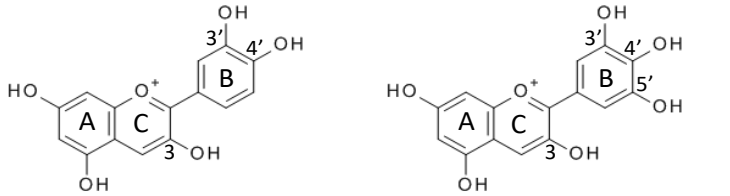
Dietary anthocyanins

Flavonoids are phenolic compounds present in plant based foods and beverages. Anthocyanins are a sub-class of flavonoid that exist in a variety of berry fruits such as blueberry, bilberry, raspberry, strawberry and other foods such as aubergine skins and black rice. Anthocyanins are what confer the red, black, purple and blue colours of anthocyanin rich foods. The daily consumption of anthocyanins is reported to range from 29 – 153 mg/d in European countries [1]. More than 90% of our intake of anthocyanins is derived from soft fruit and is therefore readily incorporated into the habitual diet. There are three main types of anthocyanins based on the number of hydroxyl groups on the B-ring; pelargonidin-type (monohydroxy), cyanidin-type (dihydroxy) and

delphinidin-type (trihydroxy) (see Figure 1). Although there are numerous substitutions (e.g. glycosylation, methylation, acylation) that give rise to a much larger number of structures, all anthocyanins are derived from these three structures, and the important B-ring metabolites will also be derived from these three structures. The major anthocyanins in human diets are the cyanidin-type and delphinidin-type.

Epidemiological evidence indicates that those consuming the highest quantities of anthocyanins are at a lower risk of developing cardiovascular disease (CVD) [2-4]. These observations are substantiated by several published reports from randomized controlled trials (RCT's) providing evidence that consumption of anthocyanin rich foods/extracts such as whole strawberries [5, 6], elderberry juice [7] and whortleberry extracts [8] have beneficial effects on biomarkers of CVD with beneficial effects on lipid profiles common across these studies.

Figure 1. Chemical structures of the two major dietary anthocyanin types, cyanidin and delphinidin. In plant foods, anthocyanins are always present as O-linked conjugates with sugars. For example, the major anthocyanin in black rice is cyanidin-3-O-glucoside. The B-ring is also important because it is the main source of gut microbiota-derived metabolites that have been shown to be accumulate in peripheral blood.



Cyanidin (dihydroxy B-ring) Delphinidin (trihydroxy B-ring)

Dyslipidaemia and atherosclerosis

Atherosclerosis is an underlying pathophysiological cause of CVD and is characterized, in part at least, by dyslipidaemia; an elevation in LDL cholesterol and triglycerides and a reduced conc. HDL cholesterol, either singly or in combination. Cholesterol is predominantly produced in the liver. LDL particles transport cholesterol away from the liver and deliver it into the peripheral tissues. Once inside the vessel wall, LDL cholesterol is susceptible to oxidation by free radicals that are produced by endothelial cells, and oxidized LDL drives the atherosclerotic process. HDL on the other hand is known to protect against the development of atherosclerosis by mediating a process called reverse cholesterol transport; a process by which cholesterol is transported from the peripheral tissues back to the liver for conversion to bile acids and excretion via the bile, thus inhibiting atherosclerosis. A critical part in the reverse cholesterol transport process is the transfer of cholesterol from cells to circulating HDL's and this function can be measured as the cholesterol efflux capacity (CEC) of serum. It is not only the levels of HDL that are important, its quality is also important. For example, paroxinase-1 (PON-1) is a protein which, in serum, is almost exclusively located on HDL. PON-1 has been the focus of research activities because of its capacity to stimulate

cholesterol efflux and protect HDL and LDL from oxidative modifications, thus inhibiting atherosclerotic development. In fact, there is a suggestion that PON-1 might be a better marker for risk of atherosclerotic CVD compared with HDL cholesterol [9]. PON1 genotype is also crucial, since it has been shown that the single nucleotide polymorphisms that alter amino acids located at position 55 (leucine/L or methionine/M) and 192 (arginine/Q or glutamine/R) of PON1 have a profound effect on the lipid peroxide hydrolytic activity of PON1 that is important in protecting LDL from oxidation, and that people with 55-M/M and 192-Q/Q genotypes gain greater protection against CVD [10]. MicroRNA's are small non-coding RNA molecules that have been shown to regulate gene expression and have been implicated in atherosclerosis and CVD risk. Several microRNA's are known to correlate with disease states such as atherosclerosis (mirs 33 and 758) and non-alcoholic fatty liver disease (mirs122, 192, 19a and 19b) [11, 12]. Other microRNA's (mirs 223, 24, 342p, 17 and 624) have been directly associated with familial hypercholesterolemia [13, 14].

Effects of anthocyanin consumption on lipids/lipoproteins and CVD risk

Berry fruit extract and anthocyanin supplementation has been shown to increase HDL cholesterol concentrations [8, 15], enhance HDL-associated PON-1 activity [16] and promote cholesterol efflux capacity [16] in subjects with hypercholesterolemia. Similarly, in a recent systematic review of RCT's [17] assessing the impact of anthocyanins on biomarkers of CVD risk, nine studies included LDL cholesterol as an outcome evaluated for statistical significance in the intervention group compared with a control group. Of these nine studies, four reported improvements in LDL cholesterol. Of note, is that these four studies were conducted in a population of individuals already hyperlipidaemic with a total cholesterol conc. ≥ 5.17mmol/L upon enrolment to the study. For example, in the trial conducted by Zhu et al [18], upon which the power calculations for the proposed study has been based, the mean total and LDL cholesterol conc. was 6.5 and 3.5 mmol/L respectively, derived from a study population recruited with a total cholesterol conc. ranging between 5.17 – 8 mmol/L at eligibility assessment.

Bile acids are synthesised from cholesterol in the liver and later transported to the small intestine for re-absorption or subsequent excretion in the faeces. The conversion of cholesterol into bile acids and subsequent faecal excretion is the final step in the reverse cholesterol transport process described above. The initial products of the bile acid synthetic pathway are referred to as primary bile acids. The action of intestinal bacterial flora on primary bile acids results in the formation of secondary bile acids. As such, the quantity and composition of bile acids is determined by the intestinal microbiota which in turn has been shown to correlate with the risk of atherosclerotic development [19, 20].Thus it is considered important to determine how intestinal microbiota relates to atherosclerotic disease markers such as bile acids, LDL/HDL cholesterol and whether their profile can be modulated by anthocyanins.

Glycaemic control and CVD risk

Epidemiological evidence also supports an association between glycaemic control and CVD risk [21]. Fructosamine is a glycosylated protein that reflects cumulative glycaemic control in humans over the short term. There is evidence that glycated proteins are increased in non-diabetic subjects [22, 23] and that the increase is associated with insulin resistance [22]. As insulin resistance progresses, increased blood glucose levels ensue. Insulin resistance is associated with abnormal lipid profiles [24]. It is well documented that ingestion of berry anthocyanins has an acute beneficial effect on blood glucose status by delaying carbohydrate digestion and inhibiting the rate of glucose absorption across the intestine [25]. However, the literature evidence for the long term effects of ingestion of berry anthocyanins on glucose and insulin status is not entirely clear.

Anthocyanins from bilberry and black rice

Bilberry fruit (*Vaccinium myrtillus L*) and black rice (*Oryza sativa L*.) are particularly rich sources of anthocyanins. Bilberry fruit is related to (albeit distinct from) the blueberry. Bilberries are reported to contain ~ 285 mg anthocyanins/100g fresh fruit and the majority of the anthocyanins are of the delphinidin-type [26]. Black rice is a special cultivar of rice and is reported to contain ~ 300 mg anthocyanins/100g [27]. Black rice contains exclusively cyanidin-type anthocyanins, mainly cyanidin (92 %) with a small amount of peonidin (8 %) which is 3-O-methyl-peonidin [28]. Thus, the anthocyanins in black rice are largely di-hydroxy B-ring anthocyanins whilst those in bilberry fruit are largely tri-hydroxy anthocyanins (see Figure 1). The relationship between anthocyanin structure and biological activity is not well understood but there is evidence to indicate that it may influence both absorption [29] and subsequent bioactivity [30].

Purpose of the proposed intervention trial

In the proposed study we aim to investigate the ability of each of the two major dietary types of anthocyanins to improve biomarkers of cardiovascular health in hyperlipidaemic individuals by assessing the effects on circulating cholesterol/lipids (total/HDL/LDL cholesterol & triglycerides) and markers of cardio-metabolic risk (blood glucose, fructosamine and insulin). We also aim to determine the effects of supplementing diets with these two anthocyanin types on bile acids and markers for lipid metabolism (CEC & PON-1 activity) and determine the relationship between diet-mediated changes in PON-1 activity and PON-1 genotype. In a subset of participants (n=24) we will also investigate the effects of dietary supplementation with bilberry (trihydroxy) and black rice (dihydroxy) anthocyanins on the expression of microRNAs that are known to be involved in atherosclerosis, non-alcoholic fatty liver disease and reverse cholesterol transport processes, and on faecal excretion of bile acids and lipids. *Should more resources become available during the course of the trial, we will also determine the effects of the interventions on cardio-metabolic disease related microRNA's as well as gut microbiota profiles in this subset of participants*.

3. Study hypotheses

- Daily consumption for 28 days of a bilberry extract delivering a 320 mg dose of anthocyanins will significantly reduce LDL cholesterol compared with a placebo control in individuals with higher than optimal plasma cholesterol concentrations.

- Daily consumption for 28 days of a black rice extract delivering a 320 mg dose of anthocyanins will significantly reduce LDL cholesterol compared with a placebo control in individuals with higher than optimal plasma cholesterol concentrations.

4. Study Objectives

Primary objective

To determine the effects of 28 days dietary supplementation with bilberry fruit and black rice-derived anthocyanins on circulating levels of LDL cholesterol.

Secondary objectives

To determine the effects of 28 days dietary supplementation with bilberry fruit and black rice derived anthocyanins on other biomarkers related to vascular function (e.g. total cholesterol, HDL cholesterol and triglycerides), and glycaemic control (glucose, insulin, fructosamine).

To investigate the effects of bilberry fruit and black rice derived anthocyanin ingestion on circulating bile acids and bile acid derivatives, PON-1 activity and cholesterol efflux capacity, and determine the relationship between diet mediated changes in Pon-1 activity and PON-1 genotype affecting PON-1 protein at positions 55 and 192.

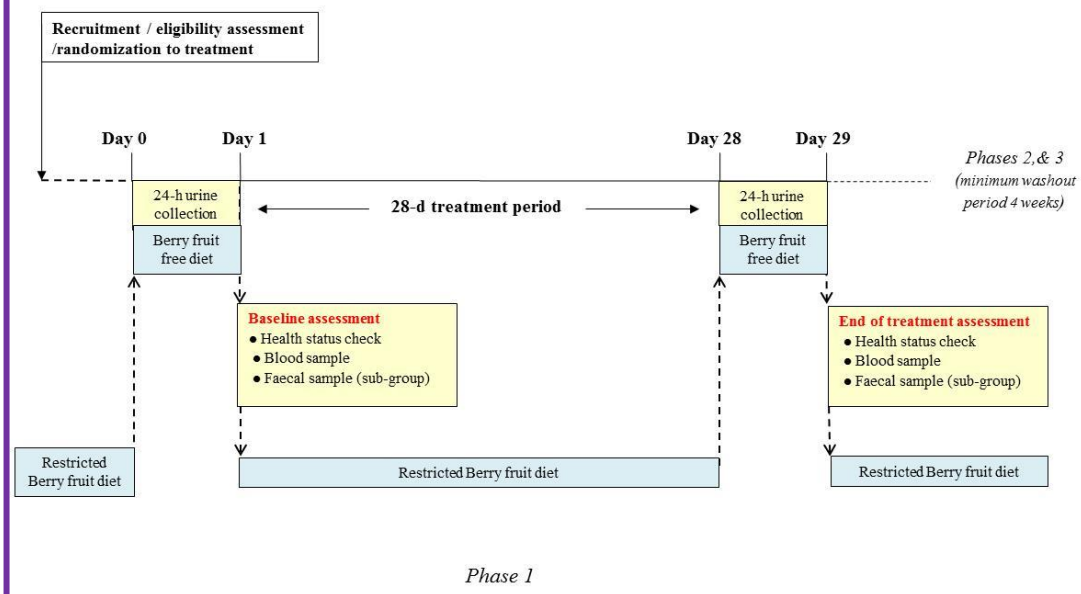
To investigate in the sub-set of participants the relationship between bilberry fruit and black rice derived anthocyanin ingestion on (i) expression of microRNAs that have been implicated in atherosclerosis, non-alcoholic fatty liver disease and reverse cholesterol transport and (ii) faecal excretion of bile acids and lipids.

Should more resources become available during the course of the trial we will also determine the effects of the interventions on gut microbiota profiles and cardio-metabolic disease related microRNA's in this subset of participants.

5. Study Design

A randomized, double blind, placebo-controlled, three-arm crossover trial (see fig 2 for overview) will be undertaken to assess the effects of (i) a bilberry extract providing 320 mg/d anthocyanins, (ii) a black rice extract providing 320 mg/d anthocyanins and, (iii) a placebo control. The test materials will be encapsulated. Each test arm will comprise a 28-day treatment period which will be identical in nature (except for the test compound consumed) and separated by a washout period of at least 4 weeks. For 2 weeks prior to commencing the first study arm and then for the duration of the entire study participants will be required to restrict berry fruit consumption to a combined maximum of 3 portions per week. However, berry fruits will be completely excluded from the diet for the 24-h period immediately preceding the pre and post intervention assessment days.

Figure 2 - Overview of study



5.1 Basic inclusion criteria

- Men and women ≥ 45 years of age
- Total cholesterol ≥ 5.0 mmol/L

5.2 Basic exclusion criteria

- Smokers (or stopped smoking for less than 3 months)
- Medical conditions that are judged to affect the primary outcome measure for this study or which may compromise the well-being of the participant e.g. diabetes. This will be assessed on a case by case basis.
- Prescribed and non-prescribed medications that may affect the primary outcome measure for this study e.g. lipid lowering therapy (this will be assessed on a case by case basis).

- Dietary supplements judged to affect the study data unless the participant is willing to discontinue them for 4 weeks preceding the start of the study and for the duration of the study. (This will be assessed on a case by case basis)
- Use foods for lowering cholesterol e.g. benecol, flora proactive (this will be assessed on a case by case basis).
- Regular/recent use of colonic irrigation or other bowel cleansing techniques.
- Intend to change the normal use of pre or probiotics during the study e.g. if participants do not take these products then we do not want them start but equally if they do take them regularly we do not want them to stop during the study. (only applies to those collecting faecal samples)
- Bowel movements ≤ 3 times per week (only applies to those collecting faecal samples)
- Gastro-intestinal diseases (excluding hiatus hernia unless symptomatic or study intervention/procedure is contra-indicated).
- Parallel participation in another research project which involves dietary intervention
- Participation in another research project which has involved blood sampling within the last four months unless the total amount of combined blood from both studies does not exceed 470 ml.
- Has donated or intends to donate blood within 16 weeks prior to or during the study period.
- Any person related to or living with any member of the study team
- Lack of capacity to provide written informed consent
- Are pregnant or have been pregnant within the last 12 months

5.3 Clinical screening exclusion criteria

- Clinical screening results deemed by the HNU Medical Advisor to be indicative of a health problem, which may compromise the well-being of the participant, or which could affect the study data.
- Depressed or elevated blood pressure measurements (<90/50 or 95/55 if symptomatic or ≥160/100 mmHg)
- BMI (Kg/m^2) <19.5 or > 40

6. Recruitment strategy

A total of 50 participants aged ≥ 45 years with a total cholesterol concentration ≥ 5.0 mmol/L at clinical screening are required to complete this trial. Based on screening data obtained from two previous trials we expect ~ 70% of otherwise eligible participants aged 45+ years to have a total cholesterol conc. ≥ 5.0 mmol/L. However, recruitment for this study will only cease once we are confident that the required number of participants will complete the study.

Potential participants meeting the basic criteria for this study will be identified using the Quadram Institute Bioscience Human Nutrition Unit (HNU) volunteer database. The HNU database contains names and contact details of people who have registered an interest in volunteering for human studies. Identification of participants from the database is carried out by the HNU senior research nurse. Those identified from the database will be sent a participant

information sheet (Annex 1) supported by a letter of invitation (Annex 2). A response slip and pre-paid envelope will be included.

We also aim to recruit potential participants via GP surgeries. We intend to seek the adoption of this study onto the National Institute of Health Research (NIHR) portfolio which will enable us to recruit via GP surgeries with the help of the co-ordinators from the Primary Research Network (PCRN) – East of England. **Identification of potential participants from** GP surgeries will be carried out by the surgery administrative staff and/or research nurses. The list containing the names of eligible patients identified on the database search will be checked by the GPs in that practice before mailing the participant an invitation letter (Annex 3), participant information sheet (Annex 1) and a pre-paid response envelope. A pack containing information about the study and copies of the study approval letters will be sent to all GP surgeries who indicate an interest in assisting with recruitment. If permitted, we will place an electronic copy of the study advertisement (Annex 4) on the website of participating GP surgeries.

In addition, poster and E-mail advertisements (Annex 4) will be placed across the Norwich Research Park (University of East Anglia (UEA), John Innes Centre (JIC), The Earlham institute, QI and other suitable locations such as social clubs, supermarkets, fitness centres etc. within the vicinity of QI (approx. 40 mile radius), inviting anyone who is interested in receiving information about the study to contact named researchers or the HNU senior research nurse. The local media (e.g. radio, newspapers/magazines etc.) may also be approached to advertise the study. We will also take advantage of social networking sites such as Facebook and twitter which will display either the ethically approved advert or be used to direct potential participants to a website where the approved advert is already displayed. Those responding to advertisements will be sent a letter of invitation (Annex 5) along with a participant information sheet (Annex 1). A response slip and pre-paid envelope will also be included.

Those participants responding positively after reading the information sheet will be invited to the HNU for an informal meeting with a member of the research team who will explain the study fully, focusing on the participant's involvement. Participants will be encouraged to ask questions at this stage prior to making any commitment. At the end of the meeting, participants will be given a minimum of 24 hours to consider whether they wish to take part in the study. During the consideration period, participants will not be contacted. If, following the consideration period they wish to participate in the study, they will be asked to contact the study scientist or HNU senior research nurse to arrange an appointment at the HNU for the pre-study clinical screening. For those individuals who are very keen to participate in the study and request to book a date for their clinical screening whilst at this meeting, this may be arranged. However, the screening appointment will be arranged for some time after the 24-hour consideration period and there will be no contact during this time.

6.1 Clinical screening

Prior to the clinical screening, participants will be reminded to bring details of any prescribed medication, herbal remedies or dietary supplements. Participants will attend the HNU following a 10 hour overnight fast but will be advised that they should drink water during the fasting period. All potential participants will be assessed for eligibility on the basis of the inclusion/exclusion criteria, eligibility questionnaire and the results of clinical laboratory tests.

On arrival at the HNU the study scientist will go through the consent form (Annex 6) with the participant and answer any questions that may arise at this stage. He/she will then be asked to sign a consent form agreeing to participate in the study. Participants will also be asked to sign a medication declaration form (Annex 7) agreeing to inform the study scientist of any medication (prescribed or not) that they ingest once they have started the trial. A copy of these forms will be given to the participant to keep. An HNU research nurse will then complete a basic eligibility questionnaire (Annex 8) measure and record blood pressure, pulse, height and weight and calculate body mass index. A 12 mL blood sample (equivalent to ~ 2 teaspoons) will be obtained and sent to Spire hospital for FBC, lipid profile (Total cholesterol, HDL & LDL cholesterol & triglycerides) and HbA1c analysis. Upon completion of the clinical screening, participants will be offered a standard HNU breakfast.

Results of the screening blood test that fall outside the standard reference ranges will be assessed by the HNU Medical Advisor (or in her absence a doctor from the UEA medical centre) who will advise whether inclusion, re-screen or exclusion is appropriate. It is possible that some minor deviations from the normal reference range will neither affect the study data nor have health implications for the participant. In this case, the medical advisor may deem inclusion the appropriate course of action and write 'satisfactory' on the blood result form. These participants will be included in the study without being informed that the results of the blood test fell outside the standard reference range. If however, a re-screen is deemed the appropriate course of action then the participant will be contacted and offered an appointment for a further blood test. This will be after an appropriate period of time as advised by the medical advisor. Those participants who display screening parameters outside the standard reference ranges on both occasions may be excluded from the study depending upon the results flagged. Participants who do not wish to be re-screened will be excluded from the study. Participants who are excluded from the study will be informed of 'flagged' results by telephone and a letter advising them to contact their GP (Annex 9). Study scientists will not inform the participant which results are flagged or advise on any course of action. If the blood test results indicate a medical problem requiring immediate attention (e.g. anaemia, raised blood glucose) the HNU medical advisor may advise the HNU senior research nurse to contact the participant directly to inform them of the results and enable them to make an appointment with their GP. The test results will be sent to the GP within 24 hours of comment by the medical advisor. Participants who meet the study criteria and whose clinical screening results are deemed satisfactory will be advised that they may continue with the study. All clinical screening results will be forwarded to the respective GP (Annex 10) supported by an appropriate letter (Annex 11; flagged results and Annex 12; normal results). Additionally, the respective GP of those participants

successfully recruited onto the study will be informed by letter of their patient's participation (annex 13).

7. Study procedure

Once successfully recruited onto the study, participants will commence the trial no later than 12 weeks from the screening date.

7.1 Participant Randomization

Participants in this study will act as their own controls in a 3-arm randomised cross-over study involving the daily ingestion of capsules containing (i) a bilberry extract providing 320 mg anthocyanins, (ii) a black rice extract providing 320 mg anthocyanins and (iii) a placebo control over a 28 day period. Each treatment will be assigned a letter (A-C) by a staff member not involved with the trial. For emergency purposes only, a member of QI staff will receive a sealed envelope containing the above letters and corresponding treatment. In the absence of an emergency the envelope will only be opened upon completion of the data analysis by the study statistician. Randomization to treatment order will be assigned as follows: There are 6 possible sequences of the letters A-C. Each sequence of letters will be listed 1-6. Each time a participant is successfully recruited onto the study, the order of intervention will be assigned by allocating the participant to the next sequence of letters from the list. Once all 6 sequences have been used the process will be repeated. The randomization sequences will be generated by the computer programme, randomization.com. The study scientist will be responsible for the randomization process and subsequent allocation of participant code to treatment sequence. However, both the study scientists, HNU research nurses and the participants will be blinded to the treatments.

7.2 Capsule preparation

Bilberry fruit and black rice extract powders will be supplied by the Beijing Gingko Group (BGG). The BGG product specification sheets for both types of extract have been supplied with this submission (Annexes 14 & 15). The bilberry and black rice extracts will be analysed at the QI to confirm the anthocyanin content of the material. This information will be used to determine the correct mass of 'active' material required to deliver the daily dose of treatments (i) and (ii). Once established, the extracts will be encapsulated in the food grade kitchen in the HNU by appropriately trained members of the study team holding certificates in Level 2 Food Safety and Catering. Members of the research team have experience in encapsulating test materials for human consumption acquired as part of the EU BACCHUS project (REC ref No: 13/EE/0393). Product specification sheets for the capsules to be used have been included with this submission (Annexes 16 & 17). Each capsule will contain a total mass not exceeding 500 mg of material which will comprise the active ingredient (bilberry or black rice extract) and pharmaceutical grade microcrystalline cellulose (*Vivapur 112*); a biologically inactive and commonly used filler in tablets and capsules (product specification sheet attached; Annex 18). The placebo capsules will contain microcrystalline cellulose only. Filled

capsules for each treatment arm will be counted into moisture resistant HDPE bottles with an integrated desiccant cap and labelled accordingly. A subset of sealed bottles containing filled capsules for each treatment and placebo arm will be sent to an accredited laboratory for microbial safety analysis prior to human consumption. All capsules will look the same. Preliminary analysis at QI of a small sample of the two extracts indicates that participants will be required to ingest four capsules per day for each of the three treatment phases. The capsules will be stored according to the manufacturer's instructions.

NOTE: As stated previously bilberries contain ~ 285 mg anthocyanins per 100 g fresh weight (USDA). Therefore, to achieve the 320mg daily dose of anthocyanins proposed in this trial, an equivalent 112 g fresh bilberry would be required. Based on an average portion size of 80 g (FSA) this is equivalent to ingesting ~ 1.5 portions of bilberries per day.

7.3 Intervention procedure

Participants will be required to restrict their intake of berry fruits to a combined maximum of 3 portions per week. The restrictions will commence 2 weeks before starting the first study phase and then continue until he/she has completed the study in its entirety (inclusive of wash-out phases). In addition, for the 24-h preceding test days at the start and end of each treatment period, berry fruits will be completely excluded from the diet. Participants will be provided with a list of restricted fruits.

Each of the three study phases will comprise a 28-day treatment period which will be identical in nature (except for the treatment consumed) and separated by a washout period of at least 4 weeks. Each treatment period is described below.

Day 0 – As mentioned above participants will already be restricted in terms of berry fruit consumption. For this 24-h period preceding day 1 they will be required to collect all urine passed and to completely exclude berry fruits from the diet. They will also be asked to fast overnight (minimum 10 hours) but will be advised to drink as much water as they need during the fasting period.

Day 1 – Fasted participants will arrive at the HNU on the morning of day 1 to undergo a baseline assessment. The sub-set of participants (n= 24) will be required to arrive with a faecal sample. Instructions on how to do this (Annex 19) will have been given. Prior to starting any procedures, participants will be asked some basic questions to establish whether or not any changes in medical conditions/medications etc. have occurred since their last visit and whether they have experienced any adverse events not already disclosed by telephone, E-mail or letter (Annexe 20). Next, the participant will be weighed, after which a 35 mL blood sample will be obtained. Participants will then consume 4 capsules (equivalent to the daily dose) containing the allocated treatment. The capsules will be ingested with 150 mL water. Participants will be required to remain in the nutrition unit for at least 20 minutes after ingestion

of the capsules. A standard HNU breakfast consisting of toast with spread and/or cereals, tea/coffee will then be served.

Prior to leaving the HNU participants will be instructed on the correct administration/storage of the capsules and supplied with the required number of capsules to be consumed over the ensuing 28 day period. Participants will also be reminded to continue restricting berry fruit consumption to a maximum of 3 portions per week. Participants will be contacted 2-weeks into the treatment period to check on progress. Regardless, they will be advised to contact a member of the study team should they become unwell or experience any problems with taking the capsules.

Day 28 – As per day 0, participants will be required to exclude all berry fruits from the diet and collect all urine passed for the 24-h period preceding day 29. They will also be asked to fast overnight (minimum 10 hours) but will be advised to drink as much water as they need during the fasting period.

Day 29 – Fasted participants will arrive at the HNU on the morning of day 29 to undergo post intervention assessment as described for D1. The sub-set of participants (n= 24) will be required to arrive with a faecal sample. A further 35 mL blood sample will be obtained. On completion of the post intervention assessment, participants will be given a standard HNU breakfast.

In the unlikely event that a participant is unable to attend the pre-scheduled d29 assessment day, a lee-way of ± 3 days is considered an acceptable time period for the purposes of re-scheduling the appointment. In the event that the original appointment is delayed, the participant will remain on the treatment until the day preceding the final assessment day. If the participant is unable to re-schedule within ± 3 days or due to unforeseen circumstances is required to interrupt the treatment period (e.g. minor illness) they will be given the opportunity to repeat the treatment at a later date.

The blood volume obtained from a participant during a single test phase is 70 ml. The total blood volume taken over the entire study is 222 ml (incl. screening sample). This will rise to 234 mL if a participant requires a repeat screening blood test. The container to be used for each of the 24-hour urine collections will contain ~ 1 g ascorbic acid (vitamin C) as an antioxidant.

Donation of faecal samples by the sub-group of participants on this trial will be done so on a voluntary basis. It will be made clear to participants that if he/she is willing to donate the sample then it is a requirement to do so at the start and end of all three treatment phases. Participants will be given the option of providing a faecal sample within 3 days preceding the start of the treatment period (D1) or ± 3 days of finishing the treatment period (D29). If they have been unable to produce a faecal sample within the time period preceding or on D29, the assessment day will continue as scheduled but the participant will be asked to continue consuming capsules until the faecal sample has been produced. Any faecal samples provided outside the d1 and d29 assessment days will be delivered to QI within 6 hours of production. Since we will be excluding participants from this aspect of the study if their habitual daily bowel movements are ≤ 3 days per week, we do not anticipate faecal sample collection to be a problem.

The number of participants required to provide faecal samples is twenty four. To ensure that we finish the trial with the required number of faecal samples we will review the situation part way through. Should there be doubt that we will reach the target number, we will take the decision to only enrol those participants willing to donate faecal samples. This is reflected in the participant information sheet. We adopted a similar approach to recruitment in a previous study (REC ref: 13/EE/03930).

7.4 Treatment compliance

Participants will consume four capsules once daily for 28-days. They will be instructed to consume the capsules in the morning time. However, in the event that they forget, the capsules may be consumed later in the day. To assist participants with compliance to treatment they will be provided with a capsule checklist (Annex 21). Each time the capsules are consumed, participants will record this on the checklist. Participants will be contacted 2-weeks into each of the treatment periods to assess progress. Compliance to treatment will be assessed from the record sheet and the number of unused capsules returned at the end of the treatment period. Participants who ingest less than 80% of the capsules over the entire treatment period may be withdrawn from the study. This will be assessed on a case-by-case basis.

7.5 Participant expenses/payments

An inconvenience payment of £125 will be given to each participant who completes the study or pro rata for non-completion; this will increase to £155 for those participants collecting faecal samples. Participants will also be reimbursed travel expenses to and from the QI (at the current QI mileage rate) or bus/train fares on production of a receipt. Participants will be notified that such payments are liable to tax. QI staff will be taxed at source.

7.6 Participant travel during treatment periods

Participants will not be able to travel during a 28-day treatment period. However, they may travel in-between treatment periods (e.g. during the washout phases of the study). The length of time between returning from abroad and commencing the next treatment will be determined on a case-by-case basis depending upon where travelled and whether or not they developed any gastro-intestinal symptoms.

8. Analytical methods

8.1 Processing and analysis of blood samples

Whole blood will be collected into EDTA, and serum separating tubes. EDTA samples will be immediately centrifuged at 2500 x g for 10 mins to obtain plasma. Samples collected into serum separating tubes will be allowed to clot

for 30 min before centrifugation at 2000 x g for 10 mins. Sub-samples of plasma and serum will aliquoted into appropriate storage tubes, frozen on dry ice and then subsequently stored at – 80 ^{0}C until analysis.

8.1.1. Analysis of lipids, glucose, fructosamine and insulin

Total/HDL/LDL cholesterol, triglycerides glucose and fructosamine will be analysed using an automated bench top clinical chemistry analyser (RX Daytona plus) according to the manufacturer's instructions. Plasma levels of apolipoprotein A1 (ApoA1) will be quantified using an ELISA kit (Cusabio Biotech Co., Wuhan, P.R. China). We will separate and quantify HDL sub-fractions using a commercial product that is based on polyacrylamide gel electrophoresis separation of the sub-fractions, such as the Lipoprint® HDL Subfractions product from Quantimetrix (http://quantimetrix.com/lipoprint-2/product-information-2/lipoprint-hdl-subfractions-100-tests/). Insulin concentrations will be determined by enzyme-linked immunosorbent assay (Elisa) according to the manufacturer's instructions.

8.1.2 Analysis of PON-1 and cholesterol efflux capacity

PON-1 activity will be measured using spectrophotometric assays to determine the rate of cleavage of a general PON1 substrate such as para-nitrophenol-acetate. In addition, the activity will be measured using a physiologically relevant lipid peroxide substrate as described by Aviram et al [31]. PON1 protein will be quantified using ELISA or western blotting using commercially available ELISA kits or anti-PON1 antibodies. The PON1 genotypes for the major single nucleotide polymorphisms at positions 192 (glutamine/Q versus arginine/R) and 55 (leucine/L versus methionine/M) and other known SNPs in PON1 will be determined using PCR reactions with specific primers with DNA obtained from whole blood as described by Browne et al. [32] and Mackness et al [10] and sequencing of the PCR products using standard sequencing techniques.

The effects of the diets on the cholesterol efflux capacity (CEC) of blood will be quantified by measuring the rate of efflux of labelled cholesterol from pre-loaded human macrophages (phorbol myristate-treated THP-1 cells) to serum samples obtained before and after the 4-week dietary intervention. Assays will be conducted using a cholesterol efflux assay available from Abcam (Product: ab196985). By quantifying the CEC of minimally processed plasma and also plasma that has been pre-processed to remove ApoB1, we will also be able to determine if the anthocyanin-mediated changes in CEC are due to changes in ApoB1, HDL-cholesterol or both. Ethylene glycol precipitation (MW=8,000; Sigma) will be used to strip serum of apoB lipoprotein according to a protocol published by Asztalos et al. [33] CEC values will also be correlated with HDL concentrations (determined in 8.1.1).

8.1.3 Analysis of bile acids (and derivatives)

Bile acids and bile acid derivatives will be analysed using an established in-house LC-MS technique. In brief, aliquots of plasma will be mixed with methanol prior to centrifugation at 17,000 x g for 10 min. The resultant supernatant will be rotary evaporated, almost to dryness, before the addition of 1 mL aqueous methanol. Samples will be passed through an SPE cartridge before applying to LC-MS.

8.1.4 Analysis of anthocyanins

Plasma anthocyanins and anthocyanin metabolites will be analysed using an established in-house extraction procedure and LC-MS technique. In brief, trifluoroacetic acid will be added to aliquots of plasma to precipitate the proteins. Post centrifugation (16,000 x g; 4°C; 15 minutes) samples of plasma will be transferred into auto-sampler vials for HPLC analysis.

8.1.5 microRNA analysis

MicroRNA's will be extracted using a miRNAeasy serum/plasma kit (Qiagen, UK) according to the manufacturer's instructions. cDNA for individual microRNA will be synthesised using a Taqman MicroRNA reverse transcription kit (Life technologies) and quantitative PCR will be carried out using Taqman MicroRNA assays for individual microRNAs (Life technologies).

8.1.6 Plasma metabolite profiling

Plasma metabolite profiles will be determined using a protocol/platform such as the Absolute/DQ® p180 kit marketed by Biocrates Life Sciences (Innsbruck, Austria) or similar. In addition, other metabolites of interest such as those in the L-carnitine / trimethylamine (TMA) / trimethylamine-N-oxide (TMAO) pathway that are prognostic of cardiovascular and metabolic diseases will be measured using targeted LC-MS or other methods as appropriate.

8.2 Processing and analysis of faecal and urine samples

Participants will be provided with a faecal collection kit consisting of an insulated container, a plastic pot, bag and clip, and ice cube bags. A description of the collection procedure is given in Annex 19. Urine will be collected in containers with ascorbic acid as previously described. Aliquots of fresh faecal matter and urine will be placed into appropriate vials and stored at – 80 ^{0}C until analysis. Portions of faecal matter will also be used to prepare faecal slurry glycerol stocks and these will be stored at -80 ^{0}C until used.

8.2.1 Analysis of bile acids and bile acid derivatives in faeces

Bile acids and bile acid derivatives from faecal samples will be analysed using established in-house LC-MS protocols.

8.2.2 Analysis of cholesterol in faeces

Faecal cholesterol will be measured using a colorimetric assay (Cell Biolabs Inc) according to the manufacturer's instructions.

8.2.3 Microbiota profiling (resources permitting)

DNA will be extracted from the faecal samples using FastDNA™ spin kits according to the manufacturer's instructions. Gut microbiota composition will be done under contract at the Earlham Institute (Norwich Research Park, UK) using Illumina MiSeq technology to undertake 16S-based rRNA gene profiling metagenomics. In addition, we will use faecal slurries to seed simple anaerobic batch colon models which will be used to (a) measure the anthocyanin metabolic capacity of the individual faecal microbiota samples which can be compared with the appearance of anthocyanin metabolites in plasma and urine samples, and (b) identify polyphenol metabolites that alter the gut microbiota profile and metabolic capacity.

8.2.4 Analysis of anthocyanins in urine

Urinary anthocyanins and anthocyanin metabolites will be analysed using an established in-house extraction procedure and LC-MS technique. In brief, an internal standard will be added to aliquots of urine before centrifugation (16,000 x g; 4°C; 15 minutes). Samples of urine will be transferred into auto-sampler vials for HPLC analysis.

8.2.5 Analysis of microbial metabolites in faeces and urine

Metabolites in faeces and urine will be determined using NMR and / or LC-MS methods. In addition, other metabolites of interest such as those in the L-carnitine / trimethylamine (TMA) / trimethylamine-N-oxide (TMAO) pathway that are prognostic of cardiovascular and metabolic diseases will be measured using targeted LC-MS or other methods as appropriate.

8.3. *Analysis of anthocyanins in berry fruit and black rice extracts*

Berry fruit and black rice powder (5 mg), will be dissolved in 1 mL acidified aqueous methanol and centrifuged at 17,000 x g for 5 mins. The supernatant will be applied to LC-MS. Chromatographic anthocyanin peak identification will be achieved by comparison of retention times with analytical standards and mass spectral data. Anthocyanins will be quantified against reference standards over the range 0-50 µg/mL.

9. Ethical considerations

9.1 Confidentiality

Participants will be assigned a unique code which will be used on all samples/data arising from this study. Only the study scientist named in the approved documentation will be able to link the code with the participant. However, the confidential code may be broken in the event of a medical emergency as deemed appropriate and necessary by the HNU senior research nurse or medical advisor. Coded information will be kept in locked cabinets at the QI.

All personal information will be kept confidential and known only to the Chief Investigator or project scientist, HNU nurses and medical advisor and the participants GP. Personal information will be kept separately to coded information in locked cabinets at the QI.

9.2 Informed consent

To ensure participants can make an informed decision as to whether or not they wish to take part on the trial they will be provided with a PIS and invited to an informal discussion about the study. Consent will be obtained by a member of staff who is GCP trained and experienced in conducting human intervention trials.

9.3 Risks and burdens

This study involves blood sampling and as such there is a risk of minor discomfort and/or bruising at the site of venepuncture. The research nurses at QI are experienced in this procedure thus minimizing this risk. The total volume of blood collected over the entire duration of the study is 222 mL, which is less than that donated at a single session with the blood transfusion service. Regardless, iron status will be measured as part of the eligibility assessment to exclude anaemia prior to participation.

This trial also involves overnight fasting. Participants will be advised to drink water during the fasting. All study sessions will be scheduled for the morning time to reduce the length of fast and participants will be offered breakfast prior to leaving the nutrition unit. At the eligibility assessment, we will also undertake an HbA1c test to exclude undiagnosed diabetic participants

As part of the eligibility assessment exercise it is possible that some blood test results are abnormal. The procedure for this eventuality is described in section 6.1. All blood results (normal or otherwise) will be forwarded to the respective GP.

On this trial we will be recruiting participants with a higher than optimal total cholesterol. Participants who are subsequently treated with lipid lowering therapies will be excluded/removed from the trial. This information is provided in the PIS.

10. Power calculations & statistical analysis

LDL Cholesterol: The primary outcome measure for this study is LDL-cholesterol. Sample size was determined based on data from a study reported by Zhu et al [18]. We have chosen to use a conservative effect size of 0.14 mmol/L for the change in LDL cholesterol. In order to detect a change (relative to placebo) in LDL cholesterol of 0.14 mmol/L at the 0.05 level of significance and with 90% power in at least one of the treatment groups, 50 participants would be needed to complete a cross-over trial. This assumes that the

standard deviations in the treatment and placebo groups are similar to that observed in the data from Zhu et al (approximately 0.5mmol/L).

MicroRNA: Sample size for the sub-set of participants was based on a study conducted by Kim et al [34]. In order to detect a change (relative to the athero-risk group) in Mir33a of 2.24 units at the 0.05 level of significance and with 90% power in the treatment group, 24 participants would be needed to complete a crossover trial. This assumes the standard deviations in the treatment and athero-risk groups are similar to that observed by Kim et al (approximately 3.24 units).

Data will be checked for normality and transformed if necessary to allow parametric statistical techniques (e.g. paired t-test, repeated measures ANOVA) to be used for analysis. Tests will be reported as significant if $P<0.05$.

11. References

1 Tennant DR, Davidson J, Day AJ: **Phytonutrient intakes in relation to European fruit and vegetable consumption patterns observed in different food surveys.** *Br J Nutr* 2014, **112:**1214-1225.

2 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:**188-196.

3 Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, Jacobs DR, Jr.: **Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women.** *Am J Clin Nutr* 2007, **85:**895-909.

4 Cassidy A, Bertoia M, Chiuve S, Flint A, Forman J, Rimm EB: **Habitual intake of anthocyanins and flavanones and risk of cardiovascular disease in men.** *Am J Clin Nutr* 2016, **104:**587-594.

5 Alvarez-Suarez JM, Giampieri F, Tulipani S, Casoli T, Di Stefano G, Gonzalez-Paramas AM, Santos-Buelga C, Busco F, Quiles JL, Cordero MD, et al: **One-month strawberry-rich anthocyanin supplementation ameliorates cardiovascular risk, oxidative stress markers and platelet activation in humans.** *J Nutr Biochem* 2014, **25:**289-294.

6 Basu A, Betts NM, Nguyen A, Newman ED, Fu D, Lyons TJ: **Freeze-dried strawberries lower serum cholesterol and lipid peroxidation in adults with abdominal adiposity and elevated serum lipids.** *J Nutr* 2014, **144:**830-837.

7 Murkovic M, Abuja PM, Bergmann AR, Zirngast A, Adam U, Winklhofer-Roob BM, Toplak H: **Effects of elderberry juice on fasting and postprandial serum lipids and low-density lipoprotein oxidation in healthy volunteers: a randomized, double-blind, placebo-controlled study.** *Eur J Clin Nutr* 2004, **58:**244-249.

8 Soltani R, Hakimi M, Asgary S, Ghanadian SM, Keshvari M, Sarrafzadegan N: **Evaluation of the Effects of Vaccinium arctostaphylos L. Fruit Extract on Serum Lipids and hs-CRP Levels and Oxidative Stress in Adult Patients with Hyperlipidemia: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial.** *Evid Based Complement Alternat Med* 2014, **2014:**217451.

9 Patra SK, Singh K, Singh R: **Paraoxonase 1: a better atherosclerotic risk predictor than HDL in type 2 diabetes mellitus.** *Diabetes Metab Syndr* 2013, **7:**108-111.

10 Mackness B, Mackness MI, Arrol S, Turkie W, Julier K, Abuasha B, Miller JE, Boulton AJ, Durrington PN: **Serum paraoxonase (PON1) 55 and 192**

polymorphism and paraoxonase activity and concentration in non-insulin dependent diabetes mellitus. *Atherosclerosis* 1998, **139:**341-349.

11 Pirola CJ, Fernandez Gianotti T, Castano GO, Mallardi P, San Martino J, Mora Gonzalez Lopez Ledesma M, Flichman D, Mirshahi F, Sanyal AJ, Sookoian S: **Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis.** *Gut* 2015, **64:**800-812.

12 Pirola CJ, Gianotti TF, Castano GO, Sookoian S: **Circulating MicroRNA-122 signature in nonalcoholic fatty liver disease and cardiovascular disease: a new endocrine system in metabolic syndrome.** *Hepatology* 2013, **57:**2545-2547.

13 Vickers KC, Landstreet SR, Levin MG, Shoucri BM, Toth CL, Taylor RC, Palmisano BT, Tabet F, Cui HL, Rye KA, et al: **MicroRNA-223 coordinates cholesterol homeostasis.** *Proc Natl Acad Sci U S A* 2014, **111:**14518-14523.

14 Vickers KC, Shoucri BM, Levin MG, Wu H, Pearson DS, Osei-Hwedieh D, Collins FS, Remaley AT, Sethupathy P: **MicroRNA-27b is a regulatory hub in lipid metabolism and is altered in dyslipidemia.** *Hepatology* 2013, **57:**533-542.

15 Kianbakht S, Abasi B, Hashem Dabaghian F: **Improved lipid profile in hyperlipidemic patients taking Vaccinium arctostaphylos fruit hydroalcoholic extract: a randomized double-blind placebo-controlled clinical trial.** *Phytother Res* 2014, **28:**432-436.

16 Zhu Y, Huang X, Zhang Y, Wang Y, Liu Y, Sun R, Xia M: **Anthocyanin supplementation improves HDL-associated paraoxonase 1 activity and enhances cholesterol efflux capacity in subjects with hypercholesterolemia.** *J Clin Endocrinol Metab* 2014, **99:**561-569.

17 Wallace TC, Slavin M, Frankenfeld CL: **Systematic Review of Anthocyanins and Markers of Cardiovascular Disease.** *Nutrients* 2016, **8**.

18 Zhu Y, Ling W, Guo H, Song F, Ye Q, Zou T, Li D, Zhang Y, Li G, Xiao Y, et al: **Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: a randomized controlled trial.** *Nutr Metab Cardiovasc Dis* 2013, **23:**843-849.

19 Sayin SI, Wahlstrom A, Felin J, Jantti S, Marschall HU, Bamberg K, Angelin B, Hyotylainen T, Oresic M, Backhed F: **Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist.** *Cell Metab* 2013, **17:**225-235.

20 Chen ML, Yi L, Zhang Y, Zhou X, Ran L, Yang J, Zhu JD, Zhang QY, Mi MT: **Resveratrol Attenuates Trimethylamine-N-Oxide (TMAO)-Induced Atherosclerosis by Regulating TMAO Synthesis and Bile Acid Metabolism via Remodeling of the Gut Microbiota.** *MBio* 2016, **7:**e02210-02215.

21 Kannel WB, McGee DL: **Diabetes and glucose tolerance as risk factors for cardiovascular disease: the Framingham study.** *Diabetes Care* 1979, **2:**120-126.

22 Sathiyapriya V, Nandeesha H, Bobby Z, Pavithran P, Selvaraj N, Rattina Dasse N: **Insulin resistance and enhanced protein glycation in men with prehypertension.** *Clinical chemistry and laboratory medicine : CCLM / FESCC* 2006, **44:**1457-1461.

23 Sathiyapriya V, Selvaraj N, Nandeesha H, Bobby Z, Agrawal A, Pavithran P: **Enhanced glycation of hemoglobin and plasma proteins is associated with increased lipid peroxide levels in non-diabetic hypertensive subjects.** *Archives of medical research* 2007, **38:**822-826.

24 Mitchell BD, Haffner SM, Hazuda HP, Valdez R, Stern MP: **The relation between serum insulin levels and 8-year changes in lipid, lipoprotein, and blood pressure levels.** *American journal of epidemiology* 1992, **136:**12-22.

25 Castro-Acosta ML, Lenihan-Geels GN, Corpe CP, Hall WL: **Berries and anthocyanins: promising functional food ingredients with postprandial glycaemia-lowering effects.** *Proc Nutr Soc* 2016, **75:**342-355.

26 USDA database for the flavonoid content of selected foods [https://www.ars.usda.gov/ARSUserFiles/80400525/Data/Flav/Flav3.2.pdf]

27 Abdel-Aal el SM, Young JC, Rabalski I: **Anthocyanin composition in black, blue, pink, purple, and red cereal grains.** *J Agric Food Chem* 2006, **54:**4696-4704.

28 Hou H: **Identification of anthocyanins isolated from black rice (Oryza sativa L.) and their**

29 Novotny JA, Clevidence BA, Kurilich AC: **Anthocyanin kinetics are dependent on anthocyanin structure.** *Br J Nutr* 2012, **107:**504-509.

30 Jing P, Bomser JA, Schwartz SJ, He J, Magnuson BA, Giusti MM: **Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth.** *J Agric Food Chem* 2008, **56:**9391-9398.

31 Aviram M, Hardak E, Vaya J, Mahmood S, Milo S, Hoffman A, Billicke S, Draganov D, Rosenblat M: **Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities.** *Circulation* 2000, **101:**2510-2517.

32 Browne RW, Koury ST, Marion S, Wilding G, Muti P, Trevisan M: **Accuracy and biological variation of human serum paraoxonase 1 activity and polymorphism (Q192R) by kinetic enzyme assay.** *Clin Chem* 2007, **53:**310-317.

33 Asztalos BF, de la Llera-Moya M, Dallal GE, Horvath KV, Schaefer EJ, Rothblat GH: **Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediated cholesterol efflux.** *J Lipid Res* 2005, **46:**2246-2253.

34 Kim SH, Kim GJ, Umemura T, Lee SG, Cho KJ: **Aberrant expression of plasma microRNA-33a in an atherosclerosis-risk group.** *Mol Biol Rep* 2016.

APPENDIX 3

LIST OF FRUITS TO RESTRICT

Fruits to exclude/restrict

No more than **3 portions per week** (total) of the fruits listed below.

Strawberries

Blackberries

Blueberry

Raspberries
Bilberry
Blackcurrants
Red currants
Whortleberry
Cherry
Chokeberry
Cranberry
Elderberry
Goji Berry
Black/red grapes

Black plums

Please remember to completely exclude these for 24 hours before your study day assessments.

APPENDIX 4

PARTICIPANT INFORMATION SHEET

Quadram Institute Bioscience

Norwich Research Park

Colney

Norwich

NR4 7UA

PARTICIPANT INFORMATION SHEET

The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults

Short title: BERI Study

You are being invited to take part in a research study at the Quadram Institute Bioscience **(formerly Institute of Food Research**). Before you decide to take part it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish.

Part 1 of this information sheet tells you the purpose of the study and what will happen if you decide to take part.

Part 2 gives you more detailed information about the conduct of the study.

Please ask if there is anything contained in the information sheet that is not clear, or if you would like more information. Take time to decide whether or not you would like to take part. This information is yours to keep. Thank you for reading this.

Study contact details:

Wendy Hollands (study manager)
(01603) 255051
Wendy.hollands@quadram.ac.uk

Aliceon Blair (HNU senior research nurse) (01603) 255305
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Part 1

What is the purpose of the study?

Regular consumption of fruit and vegetables has been shown to reduce the risk of cardiovascular disease (CVD). The protective effect may be because of compounds contained within these foods, called flavonoids. Anthocyanins are a particular group of flavonoids and are found in berry fruits (e.g. blueberry, bilberry, raspberry, and strawberry) and other foods such as aubergine skins and black rice. Anthocyanins are what give the red, black, purple and blue colours to such foods and different foods may contain different types of anthocyanins. For example, bilberry fruit contains a different type of anthocyanin to that of black rice.

There are several 'risk' factors for CVD which can be easily measured. One of the measurements that most people know about is blood cholesterol; that is good cholesterol (otherwise called HDL cholesterol), bad cholesterol (otherwise called LDL cholesterol) and triglycerides. A small number of studies have found improvements in blood cholesterol after consumption of foods rich in anthocyanins. In this study we want to compare the effects of two different types of anthocyanins (one from bilberry fruit and the other from black rice) on cholesterol status in people with higher than optimal blood cholesterol levels. We also want to investigate the effects of the different anthocyanin types on how the body processes cholesterol (called transport and metabolism) and relate this information to a specific gene involved in the cholesterol metabolism process. How we achieve all this is describe throughout this information sheet. This study will involve the consumption of capsules that contain anthocyanins that have been removed from bilberry fruit and black rice and not the whole food. It will also involve the collection of blood and urine samples and for a small sub-group of people, the collection of faecal samples.

Why have I been invited?

You have received this information because you have responded to an advertisement about the study OR you have been identified as a potentially suitable candidate either from the volunteer database held at the QI (formerly **Institute of Food Research**) human nutrition unit (HNU) or via your GP surgery.

We are aiming to recruit a total of 50 volunteers (male and female), who are aged 45 years or more with a cholesterol level at or greater than 5 mmol/L.

You will not be able to volunteer if you:

- Are a smoker (or stopped smoking for less than 3 months)
- are diabetic
- are taking medication for high cholesterol (e.g. statins)
- have a medical condition or are taking other medications (prescribed or otherwise) which may affect the study outcome (we will advise you accordingly)
- have gastro-intestinal disease (except hiatus hernia)
- take supplements judged to affect the study data (we will advise you

accordingly).

- Use foods for lowering cholesterol e.g. benecol, flora proactive (this will be assessed on an individual basis).
- Intend to change your normal use of pre or probiotics during the study e.g. if you don't take these products we don't want you to start taking them but equally if you take them regularly we don't want you to stop taking them during the study.
- Regular/recent use of colonic irrigation or other bowel cleansing techniques.
- are pregnant or have been pregnant

  within the last 12 months
- are related to someone in the study

  team (e.g. spouse, partner, immediate family member)
- have donated or intend to donate blood within 16 weeks of the first and last study samples.
- have participated in another study which has involved blood sampling within the last four months unless the total amount of combined blood from both studies does not exceed 470 mL
- are currently involved in a study which involves dietary intervention.
- the results of our screening test indicate that you are not suitable to take part in this study
- Are unable to provide written informed

  Consent
- Have a BMI less than 19.5 or more than 40 kg/m^2

Do I have to take part?

It is up to you to decide. We will describe the study in this information sheet. If, after reading it you are interested in taking part in the study, you should contact one of the researchers named on the first page of this information sheet

OR

You can complete the attached response form and return it using the pre-paid envelope enclosed. Please feel free to say no by not responding to this information. Do not worry, nobody will contact you to try and persuade you to join the study.

After you have replied to tell us you are interested in participating, a member of the study team will contact you, and you will be invited to the HNU at the QI for a talk with the study scientist/manager. An expression of interest does not commit you to taking part.

If you are on the QI HNU volunteer database, a decision to withdraw or not to take part will not affect your participation in future studies. Similarly, if you have been recruited via your GP surgery, a decision to withdraw or not to take part will not affect your care.

What will happen to me if I do take part?

If you decide to take part, your involvement in the study will last about 6 months depending upon your availability for the study visits. You will be required to visit the HNU on 8 separate occasions (2 visits before the study starts and 6 visits during the study). Each of these visits is described below. Where possible appointments will be made at your convenience but will take place on a weekday (Mon-Fri).

Pre-study talk (**Visit 1)**

This meeting will last for about one hour. The study scientist will go through this information sheet with you and answer any questions you may have. At the end of the meeting you will be given as much time as you need to decide whether or not to take part, but this will be at least 24 hours. You will not be contacted during this time. After the 24 hours if you decide to take part you will need to contact the study scientist to arrange an appointment for the next visit.

Elibility assessment /informed consent **(Visit 2)**.

This visit will last up to one and three quarter hours. You will be asked to come to the HNU between 07.30 and 10.30 hours. You will be asked to arrive having fasted for at least 10 hours. Fasting means you must not eat anything or drink anything other than plain water. During the fasting period you may drink as much water as you need.

Before we complete the eligibility assessment you will be asked to sign a consent form agreeing to take part in the study. You will also be asked to sign a medical declaration form. These forms will also be signed by the scientist/nurse and you will be given a copy of the forms to keep. After signing the consent form you are still free to withdraw from the study at any time without giving a reason.

An HNU research nurse will then complete a brief eligibility questionnaire with you and also measure and record your blood pressure, pulse rate, weight, height and body mass index (BMI). Your height and weight measurements will be used to work out your BMI. BMI is an expression of body weight which takes into account your height. A simple formula is used to calculate your BMI. If your BMI falls outside the range for this study you will be excluded from taking part.

The HNU research nurse will then take a 12ml blood sample (equivalent to 2 teaspoons) from a vein in one of your arms. The blood sample will be used to test Full Blood Count (FBC), cholesterol (total, HDL, LDL and triglycerides) and HbA1c (blood sugar). These tests will check for anything outside of the standard reference ranges, which may affect your wellbeing or the study data if you participate. However, we are looking to recruit people with a higher than optimal total cholesterol level (at or greater than 5 mmol/L). After this you will be encouraged to have breakfast (cereal, toast, tea, coffee) before you leave the HNU.

If any of your clinical results are outside the standard reference ranges, we may recommend that you speak to your GP about the results. All results outside the reference ranges are checked by the HNU medical advisor. The medical advisor will decide whether we may include you in the study, offer you the opportunity of a second screening (re-screen) or exclude you from taking part in the study. If your results fall outside the standard reference ranges on the second occasion you may be excluded from the study depending on the results flagged. We cannot tell you what your results may mean as we are not medically qualified to do so. You should not worry if your results are flagged as it may be a one off result or it may be perfectly normal for you and your GP will be able to explain them to you. Please remember these tests are performed to determine if you are suitable for the study not to find out if you are healthy.

You will be excluded from the study if the results of the test are judged by the medical advisor to be unsuitable for your participation or your cholesterol level is less than 5 mmol/L.

The HNU research nurses are experienced in carrying out clinical procedures, and if you have any questions about any of the tests, please ask one of the nurses.

You will be invited to take part in this study if the blood test from the eligibility assessment is satisfactory and you meet all the listed criteria for participation.

The study

There are three phases to this study each of which will last for a period of 29 days. The only difference between each of the three test phases is the content of the capsules consumed. These are described here.

a) 4 capsules/ day containing 320 mg bilberry anthocyanins
b) 4 capsules/day containing 320 mg black rice anthocyanins
c) 4 capsules/day containing no anthocyanins (placebo)

The dose of anthocyanins is equivalent to eating about one and a half standard portions of bilberries per day.

The order in which you consume the capsules containing each of the three test products will be 'randomly' assigned by a computer programme. When we say "randomly assigned" this means that the order is assigned by a method similar to being picked out of a hat. You will not be able to choose your order. Neither the scientist in charge of the project or you will know in which order you are consuming them. The capsules will all look the same.

Two weeks before starting the first phase of the study you will be required to restrict your intake of berry fruits to a combined maximum of 3 portions per week. A list of fruits to restrict is provided at the end of this information sheet. The restrictions will continue until you have completed the study in its entirety. A test phase is described below:

Day 0 – You will be asked to collect all urine passed (in containers which we will provide) for 24 hours before the visit on day 1. Please note that this urine bottle will contain a white powder called ascorbic acid (vitamin C) which is needed to preserve the urine. The urine container should be kept away from children and animals. You will also be asked to completely avoid eating berry fruits.

Day 1 - (**Visit 3**)

This visit will last for approximately 1 hour. You will be asked to come to the HNU between 07.30 and 10.30 hours. You will be asked to arrive having fasted for at least 10 hours. During the fasting period, you may drink as much water as you need.

After arrival and before consumption of the test capsules a nurse will weigh you and take a 35mL blood sample (equivalent to about 3 tablespoons). You will then be given 4 capsules containing one of the test products to consume. The capsules will be taken with 150 mL water (equivalent to about ½ a glass). You will be required to remain in the nutrition unit for at least 20 minutes after consuming the capsules. Breakfast (toast, cereal and tea/coffee) will then be offered.

For the next 28 days you will be asked to continue taking 4 capsules once daily. Capsules should be taken some time during the morning. However, if you forget, they can be taken in the afternoon. Capsules may be taken with water but not at the same time as food (please allow 15 minutes either side of food). If you become unwell or experience any problems with taking the capsules, then please contact a member of the study team. You will be provided with a capsule checklist and asked to tick the checklist each day the capsules are taken. Remember, you will also be restricting your berry fruit intake.

Day 28 - You will again be required to collect all urine passed for this 24-h period immediately preceding the day 29 visit. You will also be asked to completely avoid eating all berry fruits.

Day 29 (**Visit 4)**

This visit will last about 1 hour. You will be asked to come to the HNU between 07.30 and 10.30 hours. You will be asked to arrive having fasted for at least 10 hours. On this visit another 35mL blood sample will also be obtained. Afterwards, breakfast (tea/coffee, toast and/or cereal) will be provided. This completes phase 1 of the study.

The remaining 2 test phases will each be separated by a minimum of 4 weeks and there are 2 visits per test phase (**Visits 5-8)**. You will be asked to restrict your berry fruit intake to 3 portions per week during these wash-out phases and completely avoid berry fruits for 24-hours before each of the visits to the nutrition unit.

Please note: If you intend to holiday/ travel whilst taking part on the study, we will require you to remain in the UK during each of the three 28-day test phases. However, you may holiday/travel outside the UK in-between test phases.

A flowchart giving a brief outline of the study can be found on page 10.

A small sub-group of participants (24 people) will be required to provide a faecal sample at the start and end of each of the three test phases. You will be given a choice as to whether or not you donate faecal samples but you will not be able to donate if your normal bowel movements are equal to or less than 3 times per week. If, part way through the trial there is doubt that we will reach the required number of participant donations, we will take the decision to only enrol onto the study those willing to donate faecal samples. If you do volunteer to collect faecal samples you will be provided with a collection kit and instructions on how to do this.

The total amount of blood we take in a single test phase (day 1 and day 29 inclusive) is 70 ml (about 6 tablespoons). The total amount of blood we take over the whole study, including the screening sample is 222 ml. If you need to have a repeat screening blood test this will rise to 234 mL). This is about half of that which you would give during a single blood donor session.

What we do with the blood and faecal samples is described in part 2 of this information sheet.

Should you become unwell whilst on a test phase we may ask you to stop taking the capsules and repeat that test phase once you are better. This will

depend upon the nature of the illness and whether or not it will affect the study outcome.

Access to personal information

Once recruited onto the study you will be given a code number. This number will be unique to you and is used to protect your information and make your samples anonymous. Access to your personal information is restricted to the research team, HNU research nurses, HNU medical advisor and your GP. Further details about this are given in part 2 of this information sheet.

Expenses and payments

Participation in these studies is done on a voluntary basis. However, we do recognize that being involved in the study can cause you some inconvenience and there are costs associated with visiting the HNU. You will receive an inconvenience payment of £125 for participation. This will be increased to £155 if you collect faecal samples. If you are excluded from the study or you withdraw from the study the inconvenience payment you receive will be adjusted according to how much of the study you completed before exclusion/withdrawal. You will also be reimbursed travelling expenses to and from HNU on production of a receipt for buses and trains or at the current QI mileage rate for private cars.

Please note that payments are liable to tax and you are personally liable for your own tax assessment. If you are claiming state benefit, your entitlement may be affected by payments made for participating in the study. QI employees will be taxed via the payroll.

What are the risks and side effects from taking part in this study?

There can be a small amount of discomfort associated with taking blood. This may affect some people more than others, but the discomfort occurs generally only on insertion of the needle.

You may develop a small bruise at the site of the blood sample, but this will fade as with any bruise.

The HNU research nurses will be happy to answer any questions you may have about any procedures involved.

What are the potential benefits of taking part?

There are no direct benefits for you taking part in this study. However, the information we find out from this study will contribute to our understanding of the effects of anthocyanins on risk for CVD.

Will my taking part be kept confidential?

Yes. We follow Ethics and Research Governance requirements. All information about you will be handled in confidence. More details about this are included in part 2 of this information sheet.

This concludes part 1 of the information sheet.

If the information in part 1 has interested you and you are considering taking part, then it is important that you read the additional information in part 2 before making any decision.

Part 2

What if relevant new information becomes available or changes to the study are made?

If this happens we will let you know. If changes to the study are made and they impact on your participation, you will be given the new amended participant information sheet to read before being asked to sign another consent form.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any time without giving a reason. However, the study scientist will need to be informed of your decision to withdraw. You will receive payment pro rata for samples given up to the point of withdrawal. Samples collected will be kept until the study has finished and any data collected until the point of withdrawal will still be used.

What if there is a problem?

If you have a concern about any aspect of the study you should ask to speak to a member of the study team who will do their best to answer your queries. You can telephone Wendy Hollands on (01603) 255051 or the HNU senior research nurse on (01603) 255305.

If you require independent advice about the study or you are unhappy about any aspect of the study and/or wish to complain formally, you can do this through the chairperson of the QI Human Research Governance Committee (Dr Linda Harvey) on (01603) 255000.

The QI accepts responsibility for carrying out trials, and as such will give consideration to claims from participants for any harm suffered by them as a result of participating in the trial, with the exception of those claims arising out of negligence by the participant. The QI has liability insurance in respect of research work involving human volunteers.

Please note that the Institute will not fund any legal costs arising from any such action unless awarded by a court.

Will my taking part on this study be confidential?

Only personal information which is needed for the study will be collected and this will be held in the strictest confidence. Access to your personal records is restricted to the study team, the HNU nurses, HNU medical advisor and your GP. All personal information is kept in locked cupboards at the QI.

Once recruited onto the study you will be issued with a volunteer code number. This number will be used on all your personal information and samples so that nobody else will know or be able to work out that they are yours. Coded

information is also kept in a locked filing cabinet at the QI but separately to your personal information.

The data generated from study samples will be stored in a secure archive for up to fifteen years after the end of the study. It will then be destroyed.

All research is subject to inspection and audit and although your records may be accessed for this purpose any personal information remains confidential.

Please note that the QI has CCTV cameras in use for security purposes. However, provision has been made so that volunteers attending HNU will not be identified.

Will my GP be informed?

Yes. It is routine practice to inform your GP that you are taking part in a study at QI. We will also send your GP details of all your clinical screening results (blood tests, blood pressure weight and BMI measurements). Your permission will be sought and this is one of the things you are agreeing to when you sign the consent form. If any of these blood results fall outside the standard reference ranges we may recommend you speak to your GP about it. We are unable to discuss your blood results with you.

What will happen to the samples I provide?

For all participants: The 12mL blood sample you provide at visit 2 (eligibility assessment) will be sent to Spire hospital for Full Blood Count, HbA1c (blood sugar) and lipid profile (total cholesterol, HDL and LDL cholesterol and triglycerides). The purpose of this is to check for anything outside the reference ranges which may affect your well-being if you took part but also to make sure you fit the criteria for the study.

The blood samples you provide during the course of the study will be used to investigate the differences in effects between the two anthocyanin types in the capsules you consumed on (i) components associated with CVD 'risk'. These include blood sugar status (glucose and fructosamine), levels of insulin and also lipids (total cholesterol, HDL and LDL cholesterol and triglycerides); (ii) components of blood associated with how we metabolize and transport lipids within the body and the specific gene (called PON-1) associated with the cholesterol metabolism process; and (iii) bile acids (these are breakdown products of cholesterol found in blood). Blood and urine samples you provide will also be analysed for anthocyanins and the breakdown products of anthocyanins to check how much has been absorbed by the body after consumption.

The PON-1 gene is important because it is involved in preventing atherosclerosis (a cause of CVD). We want to determine if there is a

relationship between the effects of consuming anthocyanins with PON-1 activity and PON-1 genotype. There are differences between people in their PON-1 gene (called genotype). We can find out which PON-1 genotype you are, and then we will determine if there is a relationship between different PON-1 genotypes and how they responded to the anthocyanins (e.g. changes in good cholesterol). The result of this gene test has no significance for you or your family, and you will not be told your genotype.

For the sub-group (24 people): In the faecal samples you provide, we will measure the amount of bile acids and lipids that are excreted. We will also make additional measurements of something called RNA (Ribonucleic acid) in the blood samples you have already provided. In short, when your body naturally produces new proteins your DNA (Deoxyribonucleic acid) is the code used to make RNA which in turn is the code for making the new protein. By looking at the RNA we can get an idea of the proteins that were especially made as a result of the anthocyanins you consumed from the study. The RNA's we measure will be some of those specifically related to atherosclerosis (a cause of CVD) and cholesterol metabolism.

NOTE: Should more resources become available during the trial, we will determine the effect of the two anthocyanin types on other RNA's related to CVD. By using the faecal samples you provided we may also investigate the relationship between the natural gut bacteria and faecal bile acids and lipids to determine if they can be modulated by anthocyanins.

What will happen to the results of the research study?

As a volunteer you are valuable to us but we are unable to tell you any of your individual results. The general findings of the study however, will be fed back to you in the form of a talk or in the form of a letter.

Results may be published in scientific journals or presented at meetings. It is also possible that data arising from this study will be shared with other researchers to support future research.

Please note that data is presented as a whole and is anonymous. Your name will not appear anywhere in any of the results presented, shared or published.

Who is funding the study?

This study is being funded through the Food and Health Strategic Programme grant to QI.

Who has reviewed this study?

Human studies research carried out at QI is reviewed by the Human Research Governance Committee (HRGC) and a group of independent people called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity.

This study has been reviewed and approved by HRGC and a Research Ethics Committee.

What you need to tell us?

We do need you to tell us some things for your safety and for the success of the study. Some medication may affect the information we are collecting so you need to tell us if you take any medication. You will also need to tell us if you become pregnant whilst on the study. Should you become unwell during the study or after a visit to the HNU then you need to tell us.

Taking part in this study is entirely voluntary. You are free to withdraw at any time and without giving a reason.

For further information or to arrange a study appointment, please contact:

Wendy Hollands (01603) 255051 E-mail:

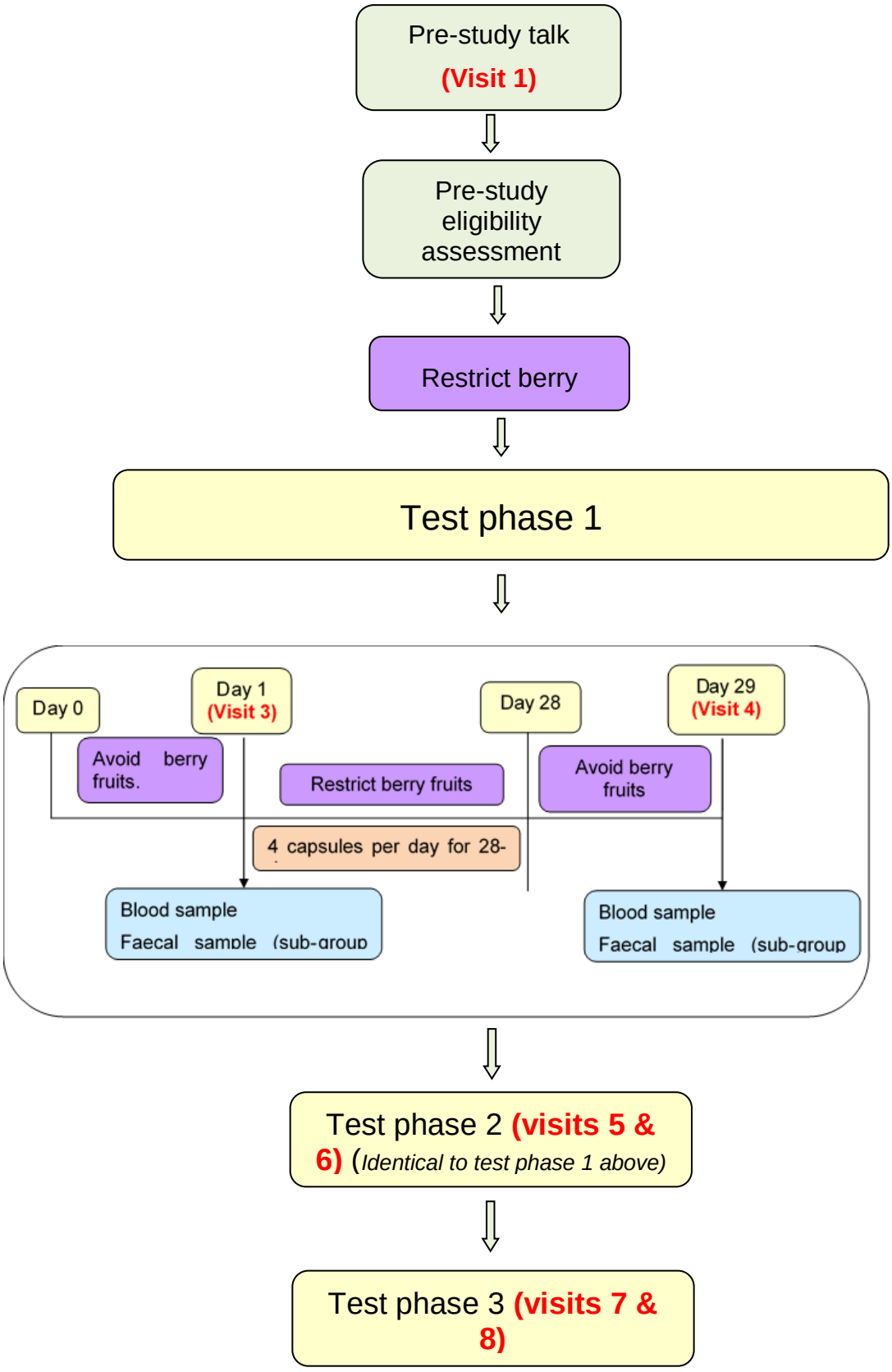
wendy.hollands@quadram.ac.uk

OR

Aliceon Blair HNU senior research nurse (01603) 255305

Alternatively you can complete the attached response slip and return this to us in the prepaid envelope provided.

Outline of study



Fruits to exclude/restrict

No more than **3 portions per week** (total) of the fruits listed below.

Strawberries

Blackberries

Blueberry

Raspberries
Bilberry
Blackcurrants
Red currants
Whortleberry
Cherry
Chokeberry
Cranberry
Elderberry
Goji Berry
Black/red grapes

Black plums

Please remember to completely exclude these for 24 hours before your study day assessments.

The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults

Short title: BERI Study

I am interested in taking part and/ or finding out more information about this study *(please complete the personal details below).*

Name: …….

Address: ……

……

……

Daytime telephone no: ……..

Evening telephone no: ……..

I am happy for a message to be left via my daytime/evening* no: **YES/NO**

**please delete as applicable*

Email address:…….

Please return this form in the FREEPOST envelope provided to:

Wendy Hollands
Quadram Institute Bioscience
FREEPOST NC 252
Norwich Research Park
Colney
NORWICH
NR4 7BR

Expressing an interest does not commit you to taking part in the study

APPENDIX 5

INVITATION LETTERS FOR PARTICIPATION IN BERI STUDY

Letter of invitation for QIB database participants

Quadram Institute Bioscience
Colney Lane
Norwich
NR4 7LU

Dear

Thank you for your interest in studies at the Quadram Institute Bioscience (formerly **Institute of Food Research)**. Please find enclosed information on a study in progress at present entitled: The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults (short title: BERI study)

Details held on the QIB Human Nutrition Unit (HNU) volunteer database suggest that you may fit the criteria for this study. If, after reading the information, you are interested in participating in this study, please complete the reply slip and return in the freepost envelope provided. Alternatively, you can contact the scientist concerned Wendy Hollands (01603) 255051.

Should your details have changed or you would prefer to no longer remain on the database please could you inform the Human Nutrition Unit on (01603) 255305.

Expressing an interest does not commit you to taking part.

Thank you very much for your time.

Yours sincerely,

Aliceon Blair

Senior Research Nurse

Letter of invitation for GP registered participants

GP Practice Headed Paper

Date

Dear (name and address),

Research study: The effects of bilberry fruit and black rice derived anthocyanins on lipid status in adults

Recently, doctors at the practice have been approached to ask if any of our patients would be interested in taking part in a research study at the Quadram Institute Bioscience in Norwich (formerly **Institute of Food Research**).

The aim of the study is to determine whether a compound in berry fruits and black rice, called anthocyanins, can have an effect on some 'risk' factors for cardiovascular disease. One main example being investigated is whether anthocyanins can lower blood cholesterol.

Our records show that you might be suitable for this research. The **Participant Information Sheet (PIS),** included with this letter, will tell you all about what taking part in the study would mean. If, after reading the PIS you would like to find out more about the study please return the reply slip (at the back of the PIS) to the study team at the Quadram Institute in the freepost envelope supplied. A member of the study team will contact you once they have received the reply slip. You can also contact a member of the study team if you wish to ask any questions about the study before you return the reply slip. The contact details for the study team are on the front of the PIS.

The study will take place in the **Human Nutrition Unit at the Quadram Institute Bioscience** (near the Norfolk and Norwich University Hospital).

Please do not send any reply slips to your GP surgery

Yours sincerely

Dr……

Letter of invitation for non-registered participants

Quadram Institute Bioscience
Norwich Research Park
Colney Lane
Norwich
NR4 7UA

Dear

Thank you for your response to the advertisement regarding a study at the Quadram Institute Bioscience (formerly **Institute of Food Research**). Please find enclosed the details of the study.

If, after reading the information, you are still interested in participating please complete the reply slip and return in the freepost envelope provided. Alternatively, you can contact the scientist/study manager **Wendy Hollands (01603) 255051** or the HNU Senior Research Nurse **Aliceon Blair (01603) 255305** as stated on the study information sheet.

Expressing an interest does not commit you to taking part.

Thank you very much for your time

Yours sincerely,

Wendy Hollands
Study Manager

APPENDIX 6

PRODUCT SPECIFICATIONS FOR BERI STUDY

PRODUCT SPECIFICATION SHEET FOR BLACK RICE EXTRACT

PRODUCT: Black Rice Extract Powder PRODUCT NO.: BR-30
ISSUE DATE: Jan. 16, 2017
SPECIFICATION NO.: 000
STARTING HERBAL MATERIAL:

BOTANICAL NAME: *Oryza sativa* L. PART OF PLANT USED: Seed

CULTIVATED/WILD: Cultivated

PRODUCTION:

SOLVENT USED: Ethanol COUNTRY OF PROCESSING: China

DRUG-TO-EXTRACT RATIO: 150-250:1 EXCIPIENTS/OTHER COMPONENTS: None ANTIOXIDANTS/PRESERVATIVES: None

PHYSICAL/CHEMICAL SPECIFICATIONS

ITEMS	SPECIFICATIONS	METHOD
CHARACTERISTICS:	Purple black powder	Visual inspection
※HEAVY METALS:	20ppm Max.	According to Eur. Ph. 2.4.8
※CADMIUM:	1ppm Max.	According to Eur. Ph. 2.4.27
※LEAD:	3ppm Max.	According to Eur. Ph. 2.4.27
※MERCURY:	0.1ppm Max.	According to Eur. Ph. 2.4.27
LOSS ON DRYING:	5.0% Max.	According to Eur. Ph. 2.8.17
ASH:	3.0% Max.	According to Eur. Ph. 2.4.16
※PESTICIDES:	In accordance to EC Regulation No. 396/2005 and modifications	
ANTHOCYANINS:		
Content of Anthocyanins by HPLC (on dry basis, expressed as Cyanidin 3-O-glucoside)	30.0% Min.	In-house method

Beijing Gingko Group
Your natural partner

Room 1706, Tower A, Building 1, Tianzuo International Center, No. 12 Zhongguancun South Avenue, Haidian District, Beijing, China, 100081

Phone: +86 (0) 10 5970 5209 Fax: +86 (0) 10 5970 5660

CYANIDIN 3-O-GLUCOSIDE:		
Content of Cyanidin 3-O-glucoside by HPLC (on dry basis)	20.0% Min.	In-house method
MICROBIOLOGY		According to Eur. Ph. 2.6.12 and 2.6.13
TOTAL PLATE COUNT:	< 5,000cfu/g	
YEAST & MOLD:	< 500cfu/g	
BILE-TOLERANT GRAM-NEGATIVE BACTERIA:	< 100cfu/g	
ESCHERICHIA COLI:	Absence/g	
SALMONELLA:	Absence/10g	
※AFLATOXINS:		According to Eur. Ph. 2.8.18
SUM OF AFLATOXIN B1, B2, G1 AND G2:	< 4ppb	
AFLATOXIN B1:	< 2ppb	
※BENZO(a)PYRENE:	< 10ppb	In-house method
※SUM OF BENZO(a)PYRENE, BENZO(a)ANTHRACENE, BENZO(a)FLUORANTHENE, CHRYSENE:	< 50ppb	In-house method

Not from Japan (Regulation (EC) No 297/2011) Not irradiated

※Periodic test (not carried out batch by batch) for which BGG tests batches randomly every year. Complies: BGG can provide a Certificate of Compliance for this test with historical data from tested batches.

STORAGE:
Preserve in tight containers, protected from light. Avoid excessive heat.

SHELF LIFE:
Three (3) years when properly stored.

PRODUCT SPECIFICATION SHEET FOR BILBERRY EXTRACT POWDER

PRODUCT: Bilberry Extract Powder (*Myrti*PRO)
PRODUCT NO.: GC-021J
ISSUE DATE: Jul. 6, 2016
SPECIFICATION NO.: 000

STARTING HERBAL MATERIAL:

BOTANICAL NAME: *Vaccinium myrtillus* L.
PART OF PLANT USED: Fresh Frozen Fruit
CULTIVATED/WILD: Wild

PRODUCTION:

SOLVENT USED: Ethanol/Water
COUNTRY OF PROCESSING: China
DRUG-TO-EXTRACT RATIO: 80-100:1
EXCIPIENTS/OTHER COMPONENTS: None
ANTIOXIDANTS/PRESERVATIVES: None

PHYSICAL/CHEMICAL SPECIFICATIONS

ITEMS	SPECIFICATIONS	METHOD
CHARACTERISTICS:	Purple black powder	Visual inspection
IDENTIFICATION:	HPLC complies	According to Eur. Ph. monograph 2394
※HEAVY METALS:	20ppm Max.	According to Eur. Ph. 2.4.8
※CADMIUM:	1ppm Max.	According to Eur. Ph. 2.4.27
※LEAD:	3ppm Max.	According to Eur. Ph. 2.4.27
※MERCURY:	0.1ppm Max.	According to Eur. Ph. 2.4.27
PH:	3.0-4.5	According to Eur. Ph. 2.2.3
LOSS ON DRYING:	5.0% Max.	According to Eur. Ph. 2.8.17
ASH:	3.0% Max.	According to Eur. Ph. 2.4.16
※PESTICIDES:	In accordance to EC Regulation No. 396/2005 and modifications	
FREE ANTHOCYANIDINS: Free Anthocyanidins by HPLC	1.0% Max.	According to Eur. Ph. monograph 2394

ANTHOCYANIDINS:		
Content of Anthocyanidins by UV (on dry basis)	25.0% Min.	In-house method
ANTHOCYANINS:		
Content of Anthocyanins by HPLC (on dry basis, expressed as Cyanidin 3-O-glucoside chloride)	36.0% Min.	According to Eur. Ph. monograph 2394

MICROBIOLOGY	According to Eur. Ph. 2.6.12 and 2.6.13
TOTAL PLATE COUNT:	< 5,000cfu/g
YEAST & MOLD:	< 500cfu/g
BILE-TOLERANT GRAM-NEGATIVE BACTERIA:	< 100cfu/g
ESCHERICHIA COLI:	Absence/g
SALMONELLA:	Absence/10g
※AFLATOXINS:	According to Eur. Ph. 2.8.18
SUM OF AFLATOXIN B1, B2, G1 AND G2:	< 4ppb
AFLATOXIN B1:	< 2ppb

GMO: This is IP Non-GMO product (Regulation (EC) No 1830/2003)
Not from Japan (Regulation (EC) No 297/2011)
Free from food allergens (Dir. 2000/13/EC and subsequent amendments)
Not irradiated

※Periodic test (not carried out batch by batch) for which BGG tests batches randomly every year. Complies: BGG can provide a Certificate of Compliance for this test with historical data from tested batches.

STORAGE:
Preserve in tight containers, protected from light. Avoid excessive heat.

SHELF LIFE:
Three (3) years when properly stored.

APPENDIX 7

An article

The Effects of Anthocyanins and Their Microbial Metabolites on The Expression and Enzyme Activities of Paraoxonase 1, an Important Marker of HDL Function

nutrients

Article

The Effects of Anthocyanins and Their Microbial Metabolites on the Expression and Enzyme Activities of Paraoxonase 1, an Important Marker of HDL unction

Hassan T Aboufarrag [1,2], Paul W Needs [1], Gerald Rimbach [3] and Paul A Kroon [1,*]

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[2] Food Science and Technology Department, Faculty of Agriculture, Alexandria University, Alexandria, 23511, Egypt
[3] Institute of Human Nutrition and Food Science, Christian-Albrechts-University of Kiel, 24118 Kiel, Germany; rimbach@foodsci.uni-kiel.de
* Correspondence: paul.kroon@quadram.ac.uk

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Abstract: High circulating HDL concentrations and measures of various HDL functions are inversely associated with cardiovascular disease (CVD) risk. Paraoxonase 1 (PON1) contributes to many of the athero-protective functions of HDL, such as promoting the reverse cholesterol transport process and reducing the levels of oxidized LDL. PON1 activities are influenced by several factors, the most important being diet and genetic polymorphisms. Reported data from randomized controlled trials have shown that anthocyanin consumption increased PON1 activity. However, the underlying molecular mechanisms by which anthocyanins increase PON1 activity are not understood. Therefore, the aim of this research was to investigate the ability of anthocyanins and their metabolites to increase *PON1* gene expression and/or enzyme activities as potential mechanisms. The effect of the two predominant dietary anthocyanins and 18 of their recently identified microbial metabolites including their phase-II conjugates on *PON1* gene expression was studied using a PON1-Huh7 stably-transfected cell line and reporter gene assay. The effects of these compounds on PON1 arylesterase and lactonase activities were investigated using two isoforms of the PON1 enzyme that are the phenotypes of the 192Q/R polymorphism. None of the compounds caused even modest changes in PON1 promoter activity ($p \geq 0.05$). Further, none of the compounds at physiological concentrations caused any significant changes in the arylesterase or lactonase activity of either of the iso-enzymes. Cyanidin reduced the lactonase activity of the PON1-R192R enzyme at high concentrations (−22%, $p < 0.001$), but not at physiologically achievable concentrations. In conclusion, none of the data reported here support the notion that anthocyanins or their metabolites affect PON1 transactivation or enzyme activities.

Keywords: polyphenols; flavonoids; phase-II metabolism; anthocyanin metabolites; human metabolism; lactonase activity; arylesterase activity; promoter activity; single nucleotide polymorphism; protocatechuic acid; phloroglucinaldehyde

1. Introduction

Many epidemiological and clinical studies suggest that the consumption of anthocyanin-rich fruit and vegetables is associated with favorable improvements in lipid profiles, specifically with reductions in LDL cholesterol and increases in HDL cholesterol [1–4]. High levels of circulating HDL cholesterol are inversely correlated with the incidence of cardiovascular disease (CVD), mainly coronary heart disease (CHD) [5–7]. However, more recent studies have suggested that the relationship between HDL and CVD markers extends beyond the concentration of HDL alone and that the function of HDL may be more important than HDL concentration in protecting against CVD [8–11]. HDL possesses a number of atheroprotective functions such as mediation of cholesterol efflux from cholesterol-loaded cells, protection against oxidation and inflammation, and promotion of nitric oxide synthesis [12]. This protective effect is largely attributed to enzymes associated with HDL, including paraoxonase 1 (PON1) [13].

PON1 is an HDL-associated enzyme secreted by the liver and found to have significant anti-oxidative and anti-inflammatory properties though its lactonase, peroxidase, and esterase activities [14]. The anti-atherogenicity of PON1 is thought to be related to its ability to hydrolyze oxidized cholesteryl esters and oxidized phospholipids and degrade hydrogen peroxide, thus protecting lipoprotein particles from any further oxidative modification [15].

Polymorphisms in the *PON1* gene can affect enzyme activities, stability, and the anti-atherogenicity of the PON1 enzyme [16–20]. Among the numerous PON1 polymorphisms in humans, the Q192R and L55M polymorphisms are the ones most associated with lipoprotein oxidation and CHD risk, and there is evidence that these polymorphisms explain a significant proportion of the differences in PON1 activity between individuals [21]. People with the 192-Q/Q genotype gain greater protection against CVD compared to those with 192-R/R PON1. The 192-Q/Q PON1 enzyme is more potent in decreasing the levels of oxidized lipids in human atherosclerotic lesions than the 192-R/R PON1 enzyme [22,23]. The PON1 L55M polymorphism has also been associated with variation in serum PON1 activity but has a weaker effect [24]. PON1 polymorphisms also affect the enzyme's substrate specificity [25]. For instance, the 192-R/R PON1 enzyme hydrolyses paraoxon approximately nine times faster than the 192-Q/Q, PON1 enzyme, while the opposite occurs with diazoxon and sarin substrates [25]. Therefore, ignoring the genetic variant could lead to a false interpretation, especially, when substrates that are strongly influenced by polymorphisms such as paraoxon are being used [26]. Therefore, it is recommended to compare PON1 levels within each different genotype/phenotype group.

Anthocyanins have been reported to increase PON1 activity. A 17.4% mean increase in PON1 arylesterase was reported in response to a 24-week intervention with a mixture of purified anthocyanins extracted from bilberry and blackcurrant (Medox™) in human participants with hypercholesterolemia compared to placebo [27]. A similar effect on serum PON1 was reported for participants who had consumed pomegranate juice for two weeks compared to a control beverage [28]. In addition, treatment of PON1-Huh7 cells with polyphenol-rich and anthocyanin-rich purple sweet potato fractions was reported to cause significant induction of PON1 promoter transactivation [29]. Other polyphenols such as quercetin, resveratrol, and catechin have also been reported to modulate PON1 activity and gene expression in vivo and in vitro [30–35].

There is growing evidence that anthocyanins are subjected to extensive metabolism, especially by the gut microbiota, producing a wide range of metabolites [36]. After consumption of penta-^{13}C-labelled cyanidin-3-glucoside (C3G), most of the given dose was recovered as breakdown products (A- and B-ring-derived phenolics), while only minor quantities of intact C3G were recovered [37,38]. Of the 35 metabolites identified in human plasma, urine, and feces in this study, hippuric acid, vanillic acid, ferulic acid, 4-

hydroxybenzaldehyde, and vanillic acid sulphate were the predominant metabolites [38]. The much higher concentrations in blood and the relative stability of anthocyanin metabolites suggest that the high bioactivity of anthocyanins is more likely to be mediated by their metabolites rather than the parent compounds. However, the biological activity of the majority of these metabolites has not been investigated.

Therefore, the aim of this study was to investigate the effects of two predominant dietary anthocyanins (C3G and delphindin-3-glucoside (D3G)), and their degradation products including their phase-II conjugates on *PON1* gene expression and PON1 arylesterase and lactonase activities. The potential for anthocyanins or their metabolites to induce PON1 expression was determined using a reporter gene assay and the effects on enzyme activities assessed using isolated PON1 isoenzymes corresponding to the major functional SNPs in humans.

2. Materials and Methods

2.1. Reporter Gene Assay

2.1.1. Cell Culture

The effect of anthocyanins and their metabolites on *PON1* gene expression was evaluated using a reporter gene assay (Bioluminescence firefly luciferase assay) in cultured hepatocytes. PON1-Huh7 was a Huh7 liver hepatoma cell line that had been stably transfected with a reporter plasmid containing 1009 bp [−1013, −4] of the *PON1* gene promoter cloned into the firefly luciferase reporter vector pGL3 basic [30,39].

PON1-Huh7 cells were kindly provided by Dr. X. Coumoul and Dr. Robert Barouki, French National Institute of Health and Medical Research (INSERM), France. PON1-Huh7 cells were maintained following the protocol as previously described [39]. Briefly, PON-Huh7 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FBS) 10% (v/v), penicillin (100 U/mL) and streptomycin (100 μg/mL), glutamine (2 mM), and G418 disulphate (100 μg/mL) (Sigma-Aldrich) at 37 °C in 5% CO_2 until reaching 80%–90% confluency, with the media changed every two days. The cells were then detached by adding trypsin-EDTA for 2–3 min at 37 °C. A complete DMEM that contained 10% FBS was then added to inhibit the trypsin. The trypsinized cell suspension was collected and the cells were either split or seeded for treatments. In 24-well plates, cells were seeded at an initial density of 0.15×10^6 cells/well and left to attach for 24 h.

2.1.2. Treatments

Following seeding the cells, the media was removed and new pre-warmed media containing anthocyanins treatment was added to the appropriate wells and incubated at 37 °C, 5% CO_2 for 48 h. The treatments were C3G, D3G, and their major known human metabolites (reported elsewhere [38]) together with their potential/predicted metabolites at 1 and 10 μM. The cells were also treated with phase-II conjugates of protocatechuic acid (PCA) and gallic acid, namely PCA-glucuronides (PCA-GlcAs, including PCA-3-glucuronid (PCA-3-GlcA) and PCA-4-glucuronide (PCA-4-GlcA), each at 1 μM), PCA-sulphates (PCA-sulphs, including PCA-3-sulphate (PCA-3-Sulph) and PCA-4-sulphate (PCA-4-Sulph), each at 1 μM), gallic acid glucuronides (GA-GlcAs, including gallic acid-3-glucuronide (GA-3-GlcA) and gallic acid-4-glucuronide (GA-4-GlcA), each at 1 μM), and methylgallates (MethGA, including 3-O-methylgallic acid (3MethGA) and 4-O-methylgallic acid (4MethGA), each at 1 μM). Figure 1 depicts the chemical structure of these metabolites. In order to investigate the interaction between anthocyanin metabolites, the cells were treated with two different mixtures of anthocyanin metabolites, PCA-Mix and GA-Mix. PCA-Mix contains C3G, PCA, PCA-4-GlcA,

PCA-3-GlcA, PCA-3-Sulph, and PCA-4-Sulph, at 1 μM each. GA-Mix contains D3G, gallic acid, GA-3-GlcA, GA-4-GlcA, 3MethGA, and 4MethGA, at 1 μM each. The vehicle control was DMSO with final concentration of 0.1%. Curcumin (20 μM) (Fisher Scientific) served as a positive control alongside the anthocyanins and their metabolite treatments. Treatments were conducted in quadruplicate and the experiments were repeated at least two times. The tested compounds were purchased from Sigma Aldrich, syringic acid was purchased from Alfa Aesar, and phloroglucinol was purchased from Across Organics. PCA conjugates and gallic acid conjugates were synthesized in-house [40]. After treatment, the luciferase activity was measured as a marker of PON1 promoter activity.

2.1.3. Luciferase Assay

Luciferase activity was measured using the Luciferase Assay System (Promega, Hampshire, UK, Cat# E1500) according to the manufacturer's instructions. Briefly, after 48 h treatment, the media was removed, and the cell layer was washed twice with cold calcium- and magnesium-free PBS. The lysis buffer was then added to the cell layer and the cells were scraped. Immediately, the lysate was collected and centrifuged for 2 min at 12,000 XG at 4 °C. After that, in 96-well white plates, 100 μL of luciferase assay reagent were added to 20 μL cell lysate. The luminescence was immediately measured over 20 s using a plate reader, FLUROstar Optima (BMG labtech, UK).

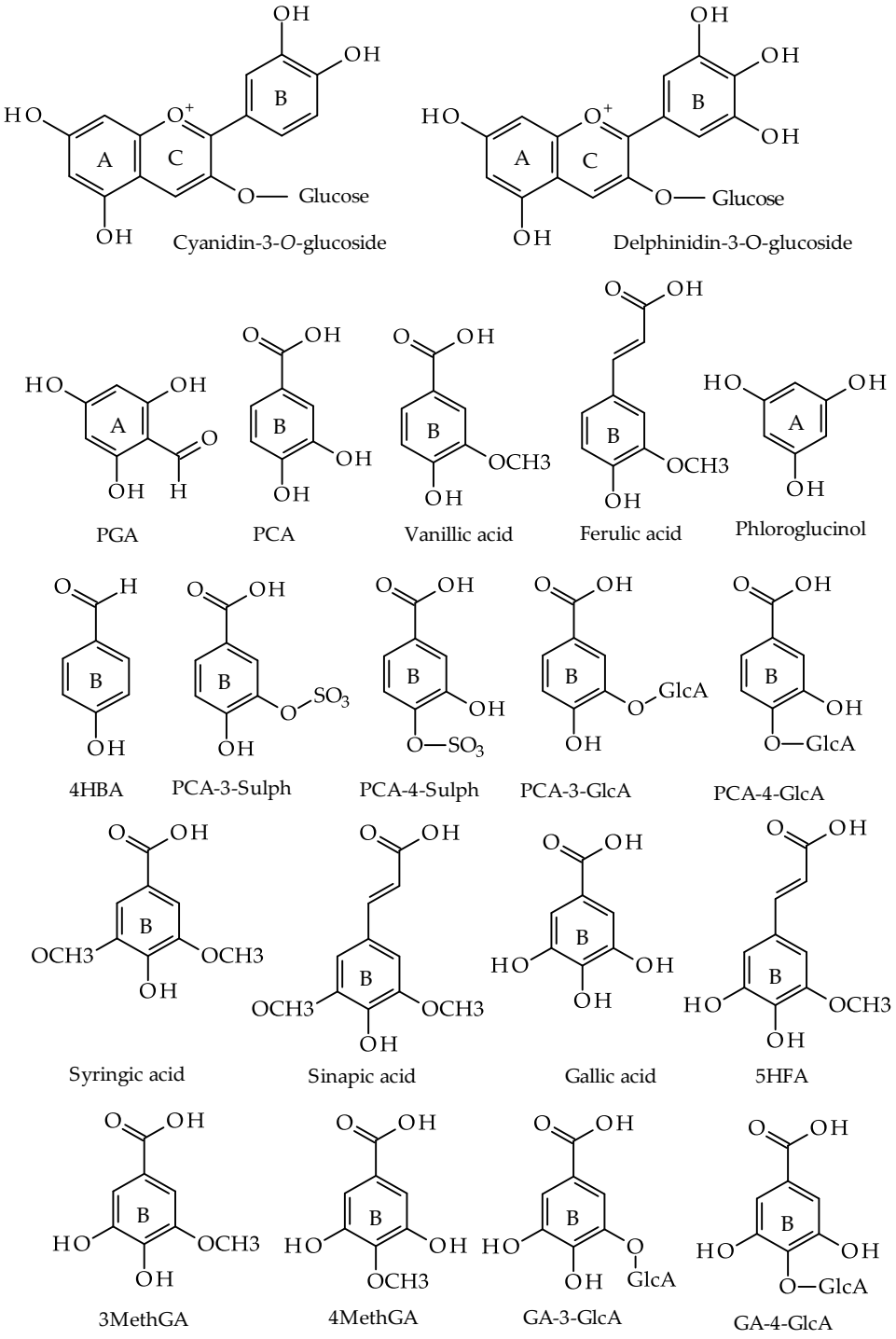

Figure 1. The chemical structures of cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, and their known and potential metabolites: phloroglucinaldehyde (PGA), protocatechuic acid (PCA), 4-hydroxybenzaldehyde (4HBA), PCA-3-sulphate (PCA-3-sulph), PCA-4-sulphate (PCA-4-Sulph), PCA-3-glucuronide (PCA-4-GlcA), PCA-4-glucuronide (PCA-4-GlcA), 3-*O*-methylgallic acid (3MethGA), 4-*O*-methylgallic acid (4MethGA), gallic-3-glucuronide (GA-4-GlcA), and gallic acid-4-glucuronide (GA-4-GlcA). AccelrysDraw (version 4.2 for windows) was used to draw the chemical structures.

The plate reader is capable of injecting the reagent automatically and can perform multi-well readings. The luminescence was expressed as total light intensity which was collected over 20 s. Results were normalized to the total cell protein content. The total protein in each sample was determined using the bicinchoninic acid (BCA)-Reducing Agent Compatible assay as per the manufacturer's protocol (Thermofisher, Paisley, UK, Cat# 23252).

2.1.4. PON1 Promoter Activity Calculation

First, the light intensity which reflects the promoter activity was normalized to the total protein content in the cell lysate.

Promoter activity = Total light intensity/total protein content (μg/mL).

Then, the fold change of PON1 promoter activity was calculated relative to the control (DMSO).

2.1.5. PON1 Enzyme Activities

The direct effect of anthocyanins on PON1 activities was measured using a commercially available purified PON1 that phenotyped into two phenotypes based on Q192R polymorphism. PON1 phenotype QQ (PON-QQ) and phenotype RR (PON-RR) were purchased from ZeptoMetrix (Buffalo NY, USA, Cat# 0801384). The treatments were prepared as described earlier and were diluted in the assay buffer. Arylesterase and lactonase PON1 activities were measured using colourimetric assays as described below.

2.1.6. PON1 Arylesterase

PON1 arylesterase activity was quantified by measuring the hydrolysis rate of p-nitrophenyl acetate (a colorless substrate) into p-nitrophenol (that has a yellow color) and measuring the increase in absorbance using a spectrophotometer [41]. PON1 arylesterase activity was measured as described elsewhere [13,42,43]. First, PON-RR and PON-QQ were diluted in assay buffer consisting of 20 mM Tris-HCl buffer, pH = 8 and 1 mM $CaCl_2$, just prior to conducting the assay. The dilution factors for PON-RR and PON-QQ were 10- and 15-fold, respectively. The assay was developed with a final volume of 200 μL in 96-well polystyrene plate. Here, 20 μL of diluted enzyme and 20 μL of treatment were mixed together with 140 μL pre-warmed assay buffer. The plate was then sealed to prevent evaporation and incubated at 37 °C for 10 min. Quickly, 20 μL of diluted substrate was added to the previous reaction mixture and the increase in absorbance was measured immediately at 410 nm for 10 min using FLUROstar Optima (BMG labtech, UK). The final concentrations of reactants were 1% PON-RR (0.63 U/mL)/0.7% PON-QQ (1.3 U/mL), 1 mM p-nitrophenyl acetate, and 1 or 10 μM anthocyanin treatments. The final concentration of DMSO was 0.1% in all treatments. The control treatment was enzyme treated with DMSO at final concentration of 0.1%. The slope of the reaction rate was calculated using the instrument software. Blanks without enzymes or treatments were used to correct for the spontaneous nonenzymatic hydrolysis of p-nitrophenyl acetate by subtracting blanks from the hydrolysis rate of treatments. The % of change was calculated relative to the control as following:

$$\%\ of\ Change\ = \frac{hydrolysis\ rate\ of\ treatment - hydrolysis\ rate\ of\ control}{hydrolysis\ rate\ of\ control}\ x\ 100$$

Treatments were conducted in triplicates and the experiments were repeated at least two times. The purity of the enzymes was determined by measuring the activity of enzymes with 100 μM of 2-hydroxyquinoline (2-HQ), the PON1-potent inhibitor (Sigma-Aldrich, Dorset, UK, Cat# **270873).**

2.1.7. PON1 Lactonase

PON1 lactonase activity was measured spectrophotometrically by monitoring the hydrolysis of the synthetic lipolactone substrate, 5-thiobutyl butyrolactone (TBBL) [44,45]. Lactonase activity was measured as described previously [46,47]. Briefly, PON-RR and PON-QQ were diluted to 50- and 80-fold, respectively, in the assay buffer that consisted of 50 mM Tris-HCl, 1 mM $CaCl_2$, 50 mM NaCl, and pH = 8. Using 96-well polystyrene plates, 20 µL of enzyme and 20 µL of treatment were mixed together with 60 µL pre-warmed assay buffer, and then 50 µL of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 4 mM) were mixed with the reaction mixture. The plate was sealed to prevent evaporation and incubated for 10 min at 37 °C. A solution of TBBL (2 mM) was prepared in pre-warmed assay buffer contain 2% acetonitrile just before use. After that, 50 µL of TBBL (2 mM) was added to the reaction mixture and the absorbance was measured immediately at 412 nm using a FLUROstar Optima plate reader (BMG labtech, UK). The final volume was 200 µL and the concentrations of reactants were 0.2% PON-RR (0.13 U/mL)/0.125% PON-QQ (0.23 U/mL), 1 mM DTNB, 0.5 mM TBBL, and 1 or 10 µM treatments. The lactonase activity was calculated as previously described for arylesterase. Treatments were conducted in quadruplicate and the experiments were repeated at least two times. The purity of the enzymes was determined by measuring the activity of enzymes with 100 µM of 2-hydroxyquinoline (2-HQ).

2.1.8. Statistical Analysis

All data and statistics were analyzed using GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla California USA, (https://www.graphpad.com/). All values are given as means ± SD. Any statistical difference between the groups was determined with one-way ANOVA coupled with Dunnett's multiple comparison test comparing all sample groups to control (DMSO 0.1%). Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Effects of Anthocyanins and Their Metabolites on PON1 Promoter Activity

The effect of anthocyanins and their metabolites on *PON1* gene expression was investigated by measuring activation of the PON1 promoter in PON1-Huh7 stably transfected cells. As shown in Figure 2A, C3G and D3G did not cause any significant change in PON1 promoter activity compared to the control (DMSO) at either 1 or 10 µM; C3G treatment resulted in a small increase but this was not significant ($p \geq 0.05$). Similarly, a large number of anthocyanin metabolites were tested, including known metabolites of anthocyanins that were reported previously from the study that used penta-^{13}C-labelled C3G [38] (Figure 2B) and a series of predicted anthocyanin metabolites. None of these metabolites (Figure 2C) or the phase-II-conjugates (Figure 2D) that were tested had any significant effect on PON1 promoter activity. In contrast to what we had predicted, syringic acid, cyanidin, 5-HFA, and hippuric acid reduced promoter activity by between 10% and 20%, although the changes were not significant (Figure 2C). In contrast, curcumin (the positive control) significantly increased PON1 promoter activity by 5.3-fold (Figure 2A–D, $p \leq 0.001$) which was consistent with previous reports [29,39,48]. This demonstrates that the model is functioning properly and sensitive to treatments. Although these data showed that anthocyanins and their metabolites did not significantly alter the promoter activity of PON1, it is possible that they may interact with PON1 in a different way, for example by activating the enzyme and increasing its activities. Therefore, the effect of anthocyanins and their metabolites on PON1 enzyme activities were investigated.

3.2. Effect of Anthocyanins and Their Metabolites on PON1 Activities

3.2.1. Establishing a Fit-for-Purpose Enzyme Assay

To avoid inaccurate enzyme activity measurements, the reaction rate should be measured within the linear stage of the enzyme reaction. To establish a linear enzyme reaction, serial dilutions of PON-RR and PON-QQ enzymes were tested with p-nitrophenyl acetate (the substrate for arylesterase) and TBBL (the substrate for lactonase). A concentration of 1% PON-RR (0.63 U/mL) and 0.7% PON-QQ (1.3 U/mL) enzymes resulted in a linear reaction of arylesterase (Figure 3A), while the linearity of the lactonase enzyme reaction was achieved with 0.2% PON-RR (0.13 U/mL) and with 0.125% PON-QQ (0.23 U/mL) (Figure 3B). The linear regression coefficient (R^2) was about 0.99, which is very close to 1 for both arylesterase and lactonase, meaning that the reaction is linear at these concentrations of PON-RR and PON-QQ. In addition, the reaction remained linear for at least 16 min for both reactions (Figure 3A and B). To make sure that the model was working and to validate that the commercial enzyme does not contain any other esterases rather than PON1, the arylesterase and lactonase activities were measured in the presence and absence of 2-hydroxyquinoline (2-HQ), a potent inhibitor of PON1. It was found that 2-HQ resulted in about 97.5% inhibition for PON-RR and PON-QQ for both activities, confirming the suitability of the model for this purpose of study (Figure 3A and B).

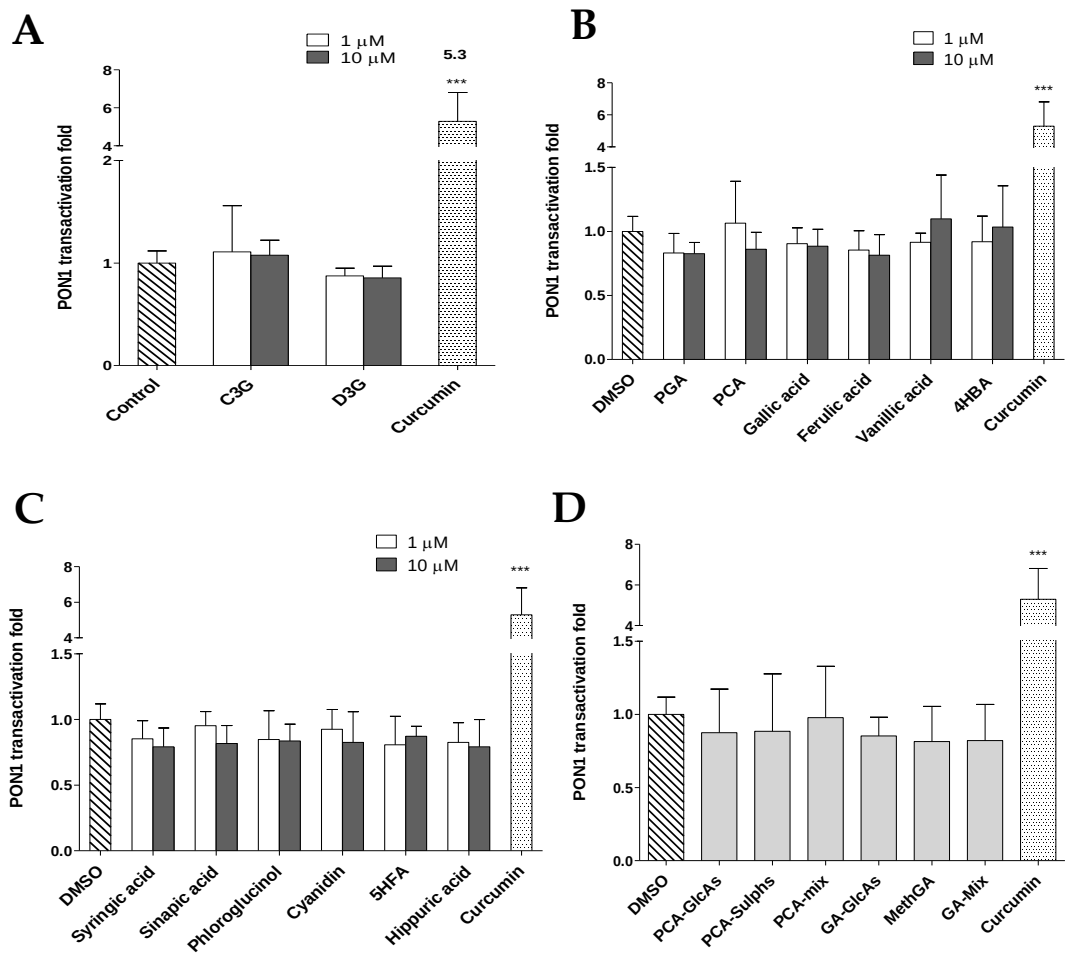

Figure 2. Effect of anthocyanin parent compounds (**A**), their known human metabolites (**B**), their potential/predicted metabolites (**C**), and phase-II conjugates (**D**) on paraoxonase 1 (PON1) promoter activity. A reporter gene assay was used to measure the promoter activity in PON1-Huh7 cells. The fold change was calculated relative to control (DMSO). Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were as follows: cyanidin-3-glucoside (C3G), delphinidin-3-glucoside (D3G), phloroglucinaldehyde (PGA), protocatechuic acid

(PCA), 4-hydroxybenzaldehyde (4HBA), 5-hydroxyferulic acid (5HFA), PCA-glucuronides (PCA-GlcAs), PCA-sulphates (PCA-Sulphs) PCA-Mix (C3G, PCA, and PCA conjugates), gallic acid glucuronides (GA-GlcAs), methylgallates (MethGA), GA-Mix (D3G, gallic acid and gallic acid conjugates), and curcumin (20 μM) which served as a positive control. Treatments were conducted in quadruplicate and the experiments were repeated at least two times.

3.2.2. Effect of Anthocyanins and Their Metabolites on PON1 Arylesterase Activity

In this study, the direct effect of anthocyanin parent compounds and their metabolites on PON1 arylesterase activity was examined using two different phenotypes, PON-RR and PON-QQ (Figure 4A–D). The findings demonstrated that neither C3G or D3G affected the arylesterase activity at either concentration of the PON-RR or PON-QQ enzymes (Figure 4A). Likewise, none of the tested metabolites either the known, the potential or the phase-II conjugates affected the arylesterase activity, except for gallic acid, phloroglucinol, and cyanidin (Figure 4B–D).

Gallic acid at the higher concentration (10 μM) significantly increased PON-QQ arylesterase (but not PON-RR) by 9% compared to the control (Figure 4B, $p \leq 0.001$). Similarly, phloroglucinol at both 1 and 10 μM increased PON-QQ (but not PON-RR) but by only 4% (Figure 4C, $p \leq 0.001$). On the other hand, the high concentration of cyanidin (10 μM) but not the low concentration slightly decreased PON-RR by 6% (Figure 4C, $p \leq 0.001$). Although statistically significant effects were observed for gallic acid, phloroglucinol and cyanidin, the effects were very modest.

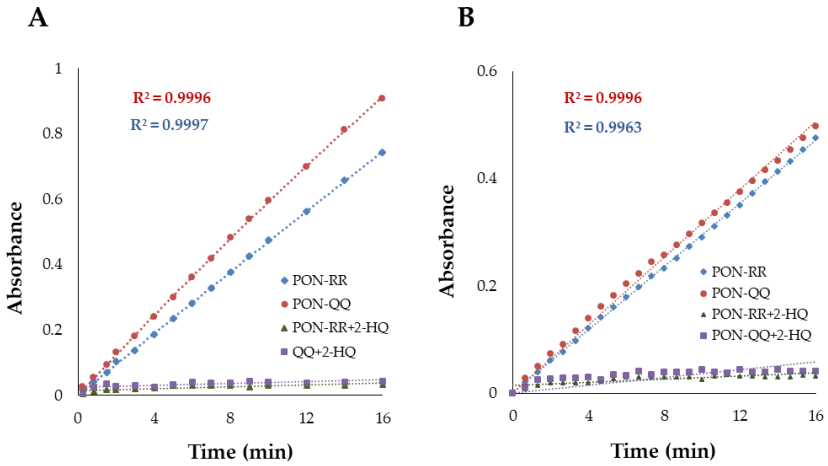

Figure 3. PON1 enzyme reaction progress curve for arylesterase (**A**) and lactonase (**B**). For arylesterase, the substrate was added to the 1% PON1 R192R phenotype (PON-RR) and to the 0.7% PON1 Q192Q phenotype (PON-QQ) with and without PON1 inhibitor (100 μM 2-hydroxyquinoline (2-HQ)). For lactonase, the substrate was added to 0.2% PON-RR and 0.125% PON-QQ with and without 100 μM 2-HQ. The enzymes were diluted in assay buffer. The absorbance was recorded over 16 min. R^2 = linear regression coefficient. R^2 was calculated using Excel software 2016.

3.2.3. Effect of Anthocyanins and Their Metabolites on PON1 Lactonase Activity.

As shown in Figure 5A–D, a few of the tested anthocyanins and their metabolites caused significant changes in PON1 lactonase activity, although the changes were not substantial. C3G had a very small but significant effect on the enzyme activity of both PON-RR and PON-QQ phenotypes. However, PON-QQ phenotype lactonase activity was affected more with increases of 11% and 9% at concentrations of 1 and 10 μM, respectively ($p \leq 0.001$), while the lactonase activity of PON-RR increased by 6% and 8% at concentrations of 1 and 10 μM of

C3G, respectively ($p \leq 0.001$, Figure 5A). On the other hand, only a modest increase (5%) in PON-RR lactonase activity was observed with D3G (1 μM) (Figure 5A, $p \leq 0.001$). Moreover, most of the tested anthocyanin metabolites did not cause significant changes, except for small changes in response to treatment with gallic acid, vanillic acid, and ferulic acid (Figure 5B, $p \leq 0.001$).

The only substantial effect was observed with cyanidin (Figure 6A and B). Unexpectedly, cyanidin at 10 μM significantly decreased PON-RR lactonase activity but not PON-QQ by 22% ($p \leq 0.001$), while the lower concentration of cyanidin did not cause a change (Figure 6A). This finding was confirmed by doing a dose-response curve for cyanidin with PON-RR enzyme. As shown in Figure 6B, cyanidin decreased lactonase activity of PON-RR in a dose-dependent manner with about 30% reduction observed at the highest concentration (20 μM cyanidin). However, the lower more physiological concentrations of cyanidin (0.1, 0.5 μM, 1 μM) did not affect PON-RR activity. On the other hand, PON-QQ appeared to respond differently when treated with cyanidin: The low concentration of cyanidin (1 μM) but not the high one slightly increased PON-QQ by 8% (Figure 6A, $p \leq 0.001$). However, it should be taken into account that the magnitude of effects where statistically significant effects were detected were modest (typically 4%–11%).

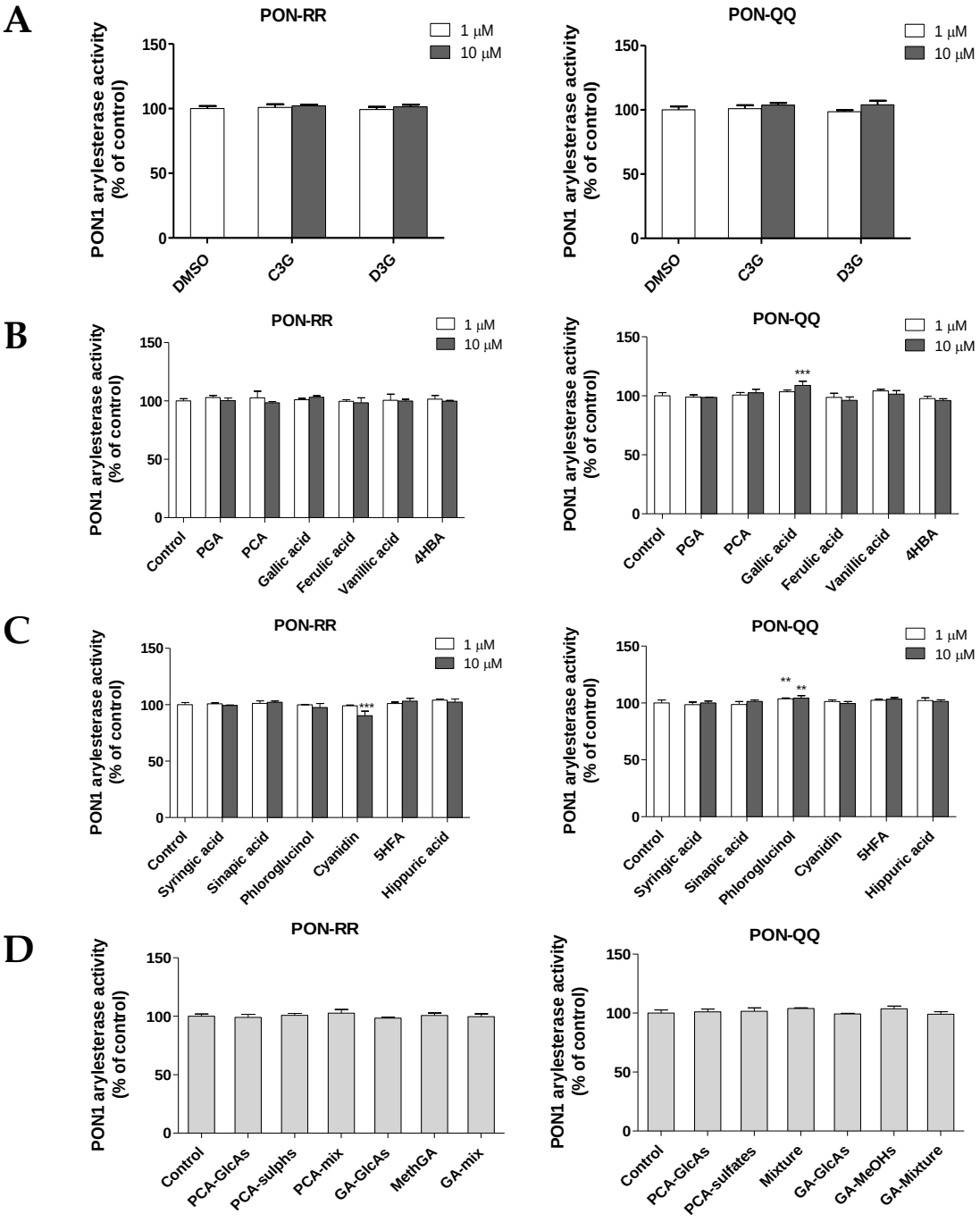

Figure 4. Effect of anthocyanins parent compounds (**A**), their known human metabolites (**B**), their potential/predicted metabolites (**C**), and phase-II-conjugates (**D**) on arylesterase activity of PON1 R192R (PON-RR) and Q192Q (PON-QQ) phenotypes. The % of change in activity was calculated relative to the control (DMSO). Data are shown as means ± SD. ** $p \leq 0.01$ and *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were: cyanidin-3-glucoside (C3G), delphinidin-3-glucoside (D3G), phloroglucinaldehyde (PGA), protocatechuic acid (PCA), 4-hydroxybenzaldehyde (4HBA), 5-hydroxyferulic acid (5HFA), PCA-glucuronides (PCA-GlcAs), PCA-sulphates (PCA-Sulphs) PCA-Mix (C3G, PCA, and PCA conjugates), gallic acid glucuronides (GA-GlcAs), methylgallates (MethGA), and GA-Mix (D3G, gallic acid, and gallic acid conjugates). Treatments were conducted in triplicates and the experiments were repeated two times.

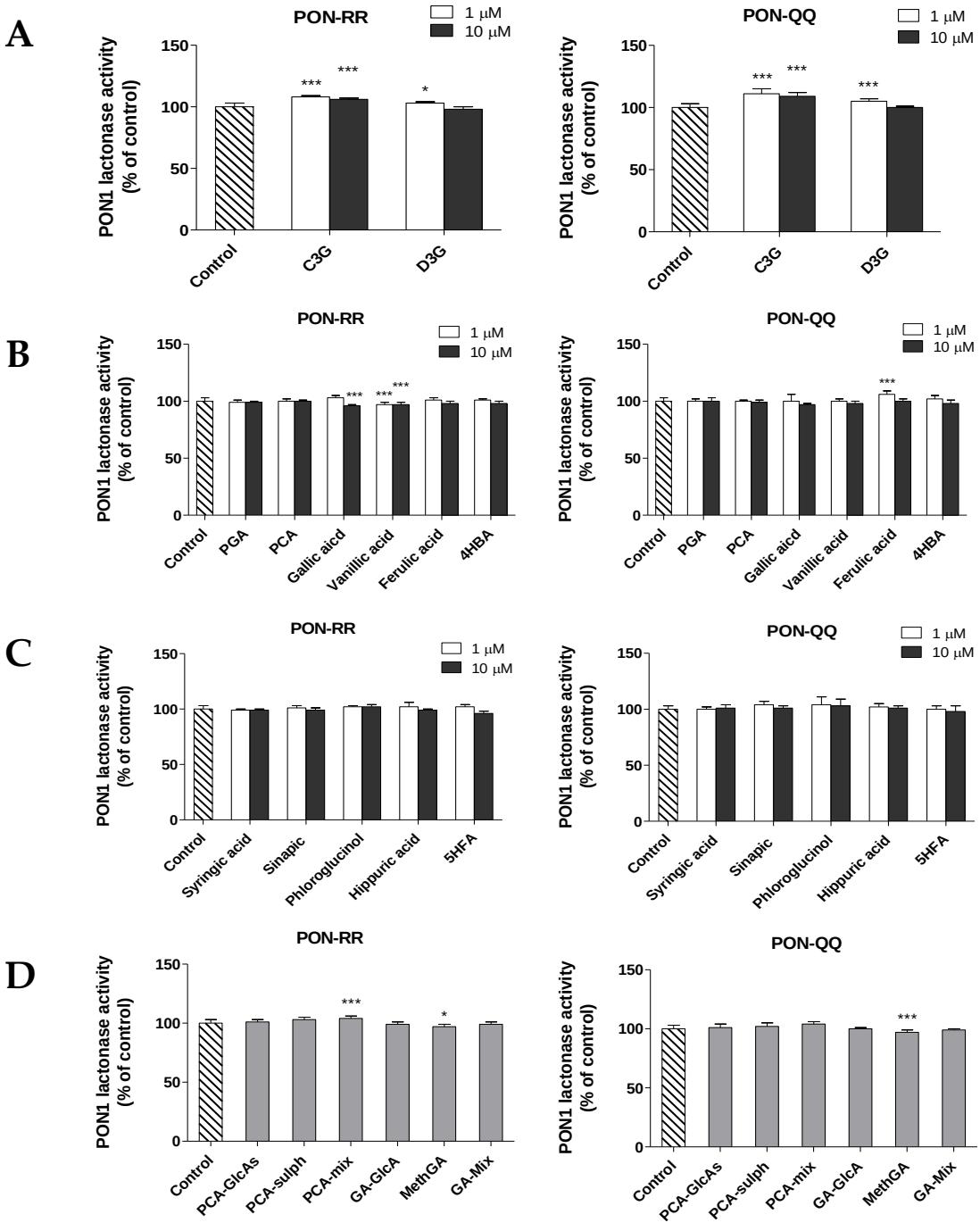

Figure 5. Effect of anthocyanins parent compounds (**A**), their known human metabolites (**B**), their potential/predicted metabolites (**C**), and phase-II conjugates (**D**) on lactonase activity of PON1 R192R (PON-RR) and Q192Q (PON-QQ) phenotypes. The % of change in activity calculated relative to the control (DMSO). Data are shown as means ± SD. * $p \leq 0.05$ and *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. Treatments were: cyanidin-3-glucoside (C3G), delphinidin-3-glucoside (D3G), phloroglucinaldehyde (PGA), protocatechuic acid (PCA), 4-hydroxybenzaldehyde (4HBA), 5-hydroxyferulic acid (5HFA), PCA-glucuronides (PCA-GlcAs), PCA-sulphates (PCA-Sulphs) PCA-Mix (C3G, PCA and PCA conjugates), gallic acid glucuronides (GA-GlcAs), methylgallates (MethGA), and GA-Mix (D3G, gallic acid and gallic acid conjugates). Treatments were conducted in triplicates and the experiments were repeated two times.

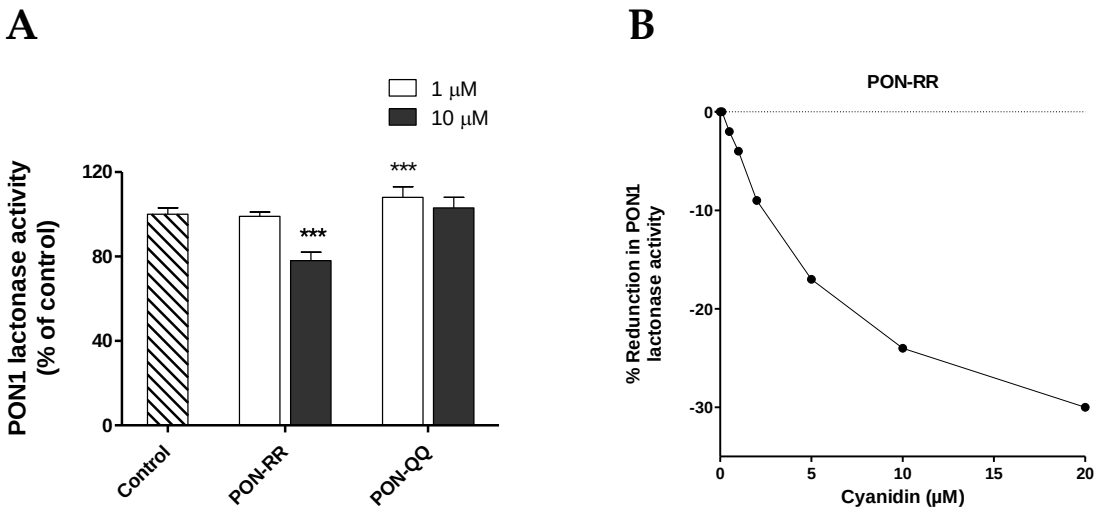

Figure 6. Effect of cyanidin on lactonase activity of PON1 R192R (PON-RR) and Q192Q (PON-QQ) phenotypes (**A**) and the dose response of PON-RR lactonase activity in the presence of cyanidin (**B**). The % of change in activity calculated relative to the control (DMSO). Data are shown as means ± SD. *** $p \leq 0.001$ as compared to control using one-way ANOVA coupled with Dunnett's multiple comparison test. % reduction in activity calculated relative to the control treatment. Treatments were conducted in triplicates and the experiments were repeated two times.

In summary, the data presented in this study do not support the notion that anthocyanins and/or their metabolites significantly affect *PON1* gene promoter activity or change the activity of PON1 enzymes. Even though some statistically significant changes were detected, the changes were very modest and probably lack clinical importance.

4. Discussion

The overall findings of the work reported here are that neither intact anthocyanins and anthocyanidins, nor their many phenolic degradation products, nor the tested phase-II-conjugates that are the forms found in human blood caused any substantive activation of the PON1 promoter or in the arylesterase or lactonase activities of the major isoforms of PON1.

Previously reported data from epidemiological studies and from dietary interventions with human participants and animal models suggests that anthocyanins have the potential to decrease the risk of CVD [49–51]. In addition, there are numerous reports describing putative biological activities of anthocyanins based on treating, for example, cultured human cells with anthocyanins, typically at supraphysiological concentrations. The vast majority of these reported in vitro studies have examined only the activity of the intact form of anthocyanins and ignored the fact that anthocyanins undergo extensive metabolism [52,53]. The unequivocal identity of the metabolites of C3G was recently established from an elegant human feeding study that used penta-^{13}C-labelled C3G that allowed the source of the metabolites (A- or B-ring of the anthocyanidin) to be established [37,38]. In addition, a number of metabolites of D3G and other trihydroxylated- and methylated-B ring anthocyanins have been reported, although anthocyanin-rich dietary sources were used instead of pure or isotope-labelled compounds [54]. The quantity of the parent (un-metabolized) ^{13}C-C3G was very low (about 0.147 μm of ^{13}C-labelled C3G), while the concentration of phenolic metabolites ranged from 0.1 to 2 μM with the cumulative concentration of metabolites reaching 10 μM [38,55] suggesting that the high bioactivity of anthocyanins is more likely to be mediated by the metabolites. However, the activities of most of these metabolites are unexplored or have only been reported at non- physiological doses, even up to 2.2 mM [56]. The present study,

therefore, investigated for the first time the possible effects of known and potential metabolites of C3G and D3G including their phase-II-conjugates relative to their parent compounds at physiologically relevant concentrations, individually and in mixtures, on *PON1* gene expression and enzyme activities. In addition, the research presented in this paper is the first to the best of our knowledge to consider the potential interaction between PON1 genotype and the dietary anthocyanins using two different phenotypes corresponding to Q192Q and R192R genotype.

As the consumption of anthocyanins or anthocyanin-rich beverages has been reported to increase serum PON1 activity in some human studies [27,28], the underlying hypothesis of this research was that the increase in PON1 activity observed in these human studies was caused by the anthocyanins or their gut microbiota metabolites interacting with *PON1* gene expression or enzyme activity and increasing the enzyme concentration and/or the enzyme activity. Here, we screened the effects not just of the anthocyanins but also of most of the major metabolites of these compounds that are products of gut microbiota metabolism. We aimed to connect the compounds to the effect and identify which compounds caused the increase in activity and/or gene expression and at which concentrations. However, the data showed that none of these compounds at physiologically relevant concentrations (1–10 μM) caused significant changes in either PON1 promoter activity or arylesterase and lactonase activities, regardless of the phenotype, except for some small effects that likely lack clinical importance. The data presented here does not support the notion that there is a direct interaction between anthocyanins and/or their metabolites with the PON1 protein or the expression of the *PON1* gene. It is possible that the increase in PON1 activity in human interventions reported previously [27,28] was due to a secondary effect(s) of consuming anthocyanins. For example, the anthocyanins/metabolites might cause changes in the gut microbiome or interact with other organs such as the liver, but it is the anthocyanin-induced changes in the function of these organs that somehow caused the levels of PON1 activity to increase.

With regard effects on expression of the *PON1* gene, we have only reported effects on the PON1 promotor activation using a reporter assay. We attempted to assess the effects of the treatments on the abundance of PON1 transcripts in the classic human hepatic cell line HepG2 using qRT-PCR. However, we were not able to detect significant expression of the *PON1* gene in HepG2 cells in our laboratory. In keeping with this observation, we were also not able to detect PON1 activity in culture supernatants (secreted PON1, Figure S1) or cell homogenates (intracellular PON1, Figure S2). Details of the experiments with HepG2 cells are provided in the supplementary information (Supplementary information 1). Others have reported the use of a PON1-Huh7 transfected cell line and reported changes in PON1 promoter activity in response to treatment with several phenolic compounds such as curcumin, resveratrol, quercetin and isorhamnetin [30,33]. Gallic acid increased PON1 promoter activity by 8.5-fold, however this was in response to a very high and non-physiological concentration of gallic acid (360 μM) [57]. Here we report the effects of a physiological concentration (1 μM) and a slightly higher concentration (10 μM), but we observed no significant effects.

Very little is currently known of the effects of direct interaction between anthocyanins or their metabolites with the PON1 enzyme. The only available report to date is concerned with the effect of naringin, which caused a decrease in PON1 arylesterase activity after incubation with isolated PON1 enzyme [13]; therefore all the data presented here are novel. The only other reports we are aware of describe the effects of phenolic compounds such as resveratrol, quercetin, punicalagin and ellagic acid on PON-1 enzyme secretion using the Huh7 cell line [30,33]. We attempted to use the HepG2 cell line to study the effect of treatments on enzyme secretion, but we were not able to detect any secreted or cell-based PON1 activity in our laboratory (Supplementary information 1).

PON1 lactonase is the physiologically relevant activity that is associated with its biological functions [26,58,59]. Impairing the lactonase activity of PON1, e.g., via mutations of

its catalytic dyad, diminished the ability of the enzyme to prevent LDL oxidation, reduced HDL-mediated cholesterol efflux, and abolished lysophosphatidylcholine (LPC) formation from phospholipid in macrophages, suggesting that the actual biological antiatherogenic functions of PON1 may be mediated by its lactonase activity [59]. However, paraoxonase and arylesterase were the only activities that had been reported previously from studies of polyphenols on PON1. To the best of our knowledge, the current study is the first to examine the effect of anthocyanins on PON1 lactonase activity and therefore provides new information regarding effects of anthocyanins on PON1 activity and by association with HDL function. Although the data presented here does not support the notion that direct interactions between anthocyanins and/or their metabolites affect the lactonase or arylesterase activity of PON1, it is not conclusive. The purified enzyme used here is not in its native environment which would include the presence of HDL and the ApoA1 protein, and this may affect the enzyme structural conformation and its stability and therefore its activity [60–64]. In theory it would be possible to use instead serum as a source of PON1. However, this may prove problematic because serum may contain other compounds such as lipoproteins that may bind with anthocyanins and conceal or inhibit their effects [65–67].

On the other hand, cyanidin decreased PON1 lactonase activity, but at super physiological concentrations and not at lower more physiological concentrations. However, the negative effect of cyanidin may be physiologically unimportant. In fact, cyanidin was not detected in serum in humans after consumption of ^{13}C-labelled C3G. Only traces of cyanidin conjugates were detected in urine in the same study suggesting that the existence of cyanidin in serum in concentrations higher than 0.1 μM is not achievable [38]. Furthermore, cyanidin is extremely unstable at high pH, with about 50% instantaneous loss of the parent structure at pH 7.4, which was lower than the pH of the assay buffer (pH = 8) used in the current study. This suggests that the reduction in PON1 lactonase activity was not mediated directly by cyanidin but may though one or more of its intermediates [68].

As far as the authors are aware, this is the first study to report the interaction between anthocyanins and their metabolites, PON1 genotype and PON1 activities. None of the previous reports that investigated the effects of anthocyanins and other polyphenols on PON1 activities have considered the association between PON1 polymorphisms and its activity [27,28]. Here we used two enzyme isoenzymes that correspond with two functional Q192R polymorphisms, PON-QQ and PON-RR. At high concentrations, cyanidin decreased PON1 lactonase activity of the PON-RR isoenzyme, but it increased the lactonase activity of PON-QQ. These observations support the notion that individuals respond differently to treatment according to their PON1 genotype, and therefore the genetic variance should be taken into account. Although both phenotypes responded similarly to the other anthocyanins/metabolites, this does not preclude that these metabolites may interact and affect the enzyme activities with other genotypes other than the Q192R genotype. In a previous nutrigenomic study, a significant association between anthocyanin intake and increased HDL levels was reported in participants with some PON1 genotypes but not with others [69]. The study investigated interactions between SNPs in the *PON1* gene and polyphenols intake on HDL and other biomarkers of CVD risk. Of 18 independent tagging SNPs that were tested, four SNPs (rs854549, rs854552, rs854571, and rs854572) showed a significant association with increased circulating HDL levels in people consuming higher quantities of polyphenols and anthocyanins [69]. At present there is no commercial source of purified isoenzymes corresponding to these genotypes to understand how these genotypes respond to anthocyanin treatments. However, undertaking a human intervention with large population and categorizing the participants into different group based on their genotype and studying the enzyme activities after anthocyanin intervention would provide a better insight into the interaction between anthocyanins, PON1 activities and PON1 genotype.

Although the data presented in this report are novel, there are several limitations. Measuring the effects of the polyphenols on promoter activity rather than gene expression (transcript levels) is one weakness. The reporter assay may not faithfully recapitulate the native chromosome environment [70]. The treatments may affect the stability of mRNA or other posttranscriptional regulators of the *PON1* gene without affecting the promotor activity, which will not be detected using the reporter assay. In addition, the effects of the anthocyanins and their metabolites on enzyme secretion has not been investigated in the current study due the authors not having a suitable model. It is possible that the treatments do not change *PON1* gene expression or the activity of the enzyme, but instead affect cell permeability or the cellular transporters that would lead to an increase in PON1 enzyme levels in serum. In our hands, HepG2 cells did not secrete detectable quantities of the enzyme, excluding its use as a suitable model for this purpose. Should a fit-for-purpose cultured cell model be available, future work should focus on the effect of anthocyanins and their metabolites on PON1 enzyme secretion to ensure that this potential mechanism of actions is also investigated.

In conclusion, the novel data presented in this study do not support the notion that anthocyanins and/or their metabolites significantly affect *PON1* gene promoter activity or change the activities of PON1 isoenzymes. Even though some statistically significant changes were detected, the changes were very modest and probably lack clinical importance. However, it is feasible that these compounds alternatively affect PON1 secretion or interact with different PON1 genotypes rather than those were reported here, and this should be considered in future studies.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, *PON1* gene expression and enzyme activity in HepG2 cell. Figure S1: Secreted PON1 arylesterase activity using HepG2 cell, Figure S2: Cell-based PON1 arylesterase activity using HepG2 cell.

Author Contribution: H.T.A. contributed in study design, performing the experiments, data analysis and interpretation, critical discussion of the results, drafting the manuscript, preparation of the final draft; P.W.N. was responsible for synthesizing polyphenols used as treatments and for editing the final manuscript; G.R. assisted with the design of the PON-HuH7 cell culture experiments and contributed to editing the final manuscript; P.A.K. provided the initial ideas and contributed to the design of experiments, data interpretation, and drafting of the manuscript.

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References

1. Hansen, A.S.; Marckmann, P.; Dragsted, L.O.; Finné Nielsen, I.L.; Nielsen, S.E.; Gronbæk, M. Effect of red wine and red grape extract on blood lipids, haemostatic factors, and other risk factors for cardiovascular disease. *Eur. J. Clin. Nutr.* **2005**, *59*, 449–455, doi:10.1038/sj.ejcn.1602107.
2. Gurrola-Díaz, C.M.; García-López, P.M.; Sánchez-Enríquez, S.; Troyo-Sanromán, R.; Andrade-González, I.; Gómez-Leyva, J.F. Effects of Hibiscus sabdariffa extract powder and preventive treatment (diet) on the lipid profiles of patients with metabolic syndrome (MeSy). *Phytomedicine* **2010**, *17*, 500–505, doi:10.1016/j.phymed.2009.10.014.
3. Qin, Y.; Xia, M.; Ma, J.; Hao, Y.; Liu, J.; Mou, H.; Cao, L.; Ling, W. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. *Am. J. Clin. Nutr.* **2009**, *90*, 485–492, doi:10.3945/ajcn.2009.27814.

4. Rodriguez-Mateos, A.; Heiss, C.; Borges, G.; Crozier, A. Berry (Poly)phenols and Cardiovascular Health. *J. Agric. Food Chem.* **2014**, *62*, 3842–3851, doi:10.1021/jf403757g.
5. Wilson, P.W.; Abbott, R.D.; Castelli, W.P. High density lipoprotein cholesterol and mortality. The Framingham Heart Study. *Arterioscler. An. Off. J. Am. Hear. Assoc. Inc.* **1988**, *8*, 737–741, doi:10.1161/01.ATV.8.6.737.
6. Shah, P.K.; Amin, J. Low high density lipoprotein level is associated with increased restenosis rate after coronary angioplasty. *Circulation* **1992**, *85*, 1279–1285, doi:10.1161/01.cir.85.4.1279.
7. Gordon, D.J.; Probstfield, J.L.; Garrison, R.J.; Neaton, J.D.; Castelli, W.P.; Knoke, J.D.; Jacobs, D.R.; Bangdiwala, S.; Tyroler, H.A. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* **1989**, *79*, 8–15, doi:10.1161/01.cir.79.1.8.
8. Haas, M.J.; Mooradian, A.D. Regulation of high-density lipoprotein by inflammatory cytokines: Establishing links between immune dysfunction and cardiovascular disease. *Diabetes. Metab. Res. Rev.* **2010**, *26*, 90–99, doi:10.1002/dmrr.1057.
9. Raffield, L.M.; Cox, A.J.; Hsu, F.C.; Ng, M.C.Y.; Langefeld, C.D.; Carr, J.J.; Freedman, B.I.; Bowden, D.W. Impact of HDL genetic risk scores on coronary artery calcified plaque and mortality in individuals with type 2 diabetes from the Diabetes Heart Study. *Cardiovasc. Diabetol.* **2013**, *12*, 95–105, doi:10.1186/1475-2840-12–95.
10. Shah, P.K. Jekyll and Hyde of HDL: A lipoprotein with a split personality. *Eur. Heart, J.* **2013**, *34*, 3531–3534, doi:10.1093/eurheartj/eht382.
11. Ikhlef, S.; Berrougui, H.; Simo, O.K.; Zerif, E.; Khalil, A. Human paraoxonase 1 overexpression in mice stimulates HDL cholesterol efflux and reverse cholesterol transport. *PLOS ONE* **2017**, *12*, doi:10.1371/journal.pone.0173385.
12. Rosenson, R.S.; Brewer, H.B.; Davidson, W.S.; Fayad, Z.A.; Fuster, V.; Goldstein, J.; Hellerstein, M.; Jiang, X.C.; Phillips, M.C.; Rader, D.J.; et al. Cholesterol efflux and atheroprotection: Advancing the concept of reverse cholesterol transport. *Circulation* **2012**, *125*, 1905–1919, doi:10.1161/circulationaha.111.066589.
13. Mahooz, A.; Rashidi, M.-R.; Nouri, M. Naringenin is an inhibitor of human serum paraoxonase (PON1): An in vitro study. *J. Clin. Lab. Anal.* **2011**, *25*, 395–401, doi:10.1002/jcla.20490.
14. Gugliucci, A.; Menini, T. Paraoxonase 1 and HDL maturation. *Clin. Chim. Acta* **2015**, *439*, 5–13, doi:10.1016/j.cca.2014.09.016.
15. Sumegová, K.; Nagyová, Z.; Waczulíková, I.; Žitňanová, I.; Ďuračková, Z.; Sumegova, K.; Nagyova, Z.; Waczulikova, I.; Zitnanova, I.; Durackova, Z.L.B.-S.; et al. Activity of Paraoxonase 1 and Lipid Profile in Healthy Children. *Physiol. Res.* **2007**, *56*, 351–357.
16. Turgut Cosan, D.; Colak, E.; Saydam, F.; Yazıcı, H.U.; Degirmenci, I.; Birdane, A.; Colak, E.; Gunes, H.V. Association of paraoxonase 1 (PON1) gene polymorphisms and concentration with essential hypertension. *Clin. Exp. Hypertens.* **2016**, *38*, 602–607, doi:10.3109/10641963.2016.1174255.
17. ME, L.; YC, L.; RT, L.; YS, W.; Hsi, E.; HF, L.; KC, C.; SH, J.; Liu, M.-E.; Liao, Y.-C.; et al. A functional polymorphism of PON1 interferes with microRNA binding to increase the risk of ischemic stroke and carotid atherosclerosis. *Atherosclerosis* **2013**, *228*, 161–167, doi:10.1016/j.atherosclerosis.2013.01.036.
18. Brophy, V.H.; Jampsa, R.L.; Clendenning, J.B.; McKinstry, L.A.; Jarvik, G.P. Effects of 5′ regulatory-region polymorphisms on paraoxonase-gene (PON1) expression. *Am. J. Hum. Genet.* **2001**, *68*, 1428–1436, doi:10.1086/320600 LB-Brophy2001.
19. Campo, S.; Sardo, M.A.; Trimarchi, G.; Bonaiuto, M.; Fontana, L.; Castaldo, M.; Bonaiuto, A.; Saitta, C.; Bitto, A.; Manduca, B.; et al. Association between serum paraoxonase (PON1) gene promoter T(-107)C polymorphism, PON1 activity and HDL levels in healthy Sicilian octogenarians. *Exp. Gerontol.* **2004**, *39*, 1089–1094, doi:10.1016/j.exger.2004.03.017.
20. Santos, F.G.; Becker, M.K.; Corrêa, V.S.; Garcia, D.N.; Vale, S.C.; Crespo-Ribeiro, J.A.; Barros, C.C.; Schneider, A. The effect of the paraoxonase 1 (PON1) T(-107)C polymorphism on serum PON1 activity in women is dependent on fatty acid intake. *Nutr. Res.* **2016**, *36*, 9–15, doi:10.1016/j.nutres.2015.10.008.

21. Gupta, N.; Singh, S.; Maturu, V.N.; Sharma, Y.P.; Gill, K.D. Paraoxonase 1 (PON1) polymorphisms, haplotypes and activity in predicting CAD risk in North-West Indian Punjabis. *PLoS ONE* **2011**, *6*, e17805, doi:10.1371/journal.pone.0017805.
22. Aviram, M.; Hardak, E.; Vaya, J.; Mahmood, S.; Milo, S.; Hoffman, A.; Billicke, S.; Draganov, D.; Rosenblat, M. Human Serum Paraoxonases (PON1) Q and R Selectively Decrease Lipid Peroxides in Human Coronary and Carotid Atherosclerotic Lesions. *Circulation* **2000**, *101*, 2510–2517, doi:10.1161/01.cir.101.21.2510.
23. Mackness, B.; Mackness, M.I.; Arrol, S.; Turkie, W.; Julier, K.; Abuasha, B.; Miller, J.E.; Boulton, A.J.; Durrington, P.N.; B., M.; et al. Serum paraoxonase (PON1) 55 and 192 polymorphism and paraoxonase activity and concentration in non-insulin dependent diabetes mellitus. *Atherosclerosis* **1998**, *139*, 341–349, doi:10.1016/S0021-9150(98)00095–1.
24. Quillen, E.E.; Rainwater, D.L.; Dyer, T.D.; Carless, M.A.; Curran, J.E.; Johnson, M.P.; Göring, H.H.H.; Cole, S.A.; Rutherford, S.; MacCluer, J.W.; et al. Novel Associations of Nonstructural Loci with Paraoxonase Activity. *J. Lipids* **2012**, *2012*, 1–7, doi:10.1155/2012/189681.
25. Brophy, V.H.; Hastings, M.D.; Clendenning, J.B.; Richter, R.J.; Jarvik, G.P.; Furlong, C.E. Polymorphisms in the human paraoxonase (PON1) promoter. *Pharmacogenetics* **2001**, *11*, 77–84, doi:10.1097/00008571-200102000–00009.
26. Ceron, J.J.; Tecles, F.; Tvarijonaviciute, A. Serum paraoxonase 1 (PON1) measurement: An update. *BMC Vet. Res.* **2014**, *10*, 74, doi:10.1186/1746-6148-10–74.
27. Zhu, Y.; Huang, X.; Zhang, Y.; Wang, Y.; Liu, Y.; Sun, R.; Xia, M. Anthocyanin supplementation improves HDL-Associated paraoxonase 1 activity and enhances cholesterol efflux capacity in subjects with hypercholesterolemia. *J. Clin. Endocrinol. Metab.* **2014**, *99*, 561–569, doi:10.1210/jc.2013-2845.
28. Aviram, M.; Rosenblat, M.; Gaitini, D.; Nitecki, S.; Hoffman, A.; Dornfeld, L.; Volkova, N.; Presser, D.; Attias, J.; Liker, H.; et al. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clin. Nutr.* **2004**, *23*, 423–433, doi:10.1016/j.clnu.2003.10.002.
29. Esatbeyoglu, T.; Rodríguez-Werner, M.; Schlösser, A.; Winterhalter, P.; Rimbach, G. Fractionation, enzyme inhibitory and cellular antioxidant activity of bioactives from purple sweet potato (Ipomoea batatas). *Food Chem.* **2017**, *221*, 447–456, doi:10.1016/j.foodchem.2016.10.077.
30. Boesch-Saadatmandi, C.; Egert, S.; Schader, C.; Coumoul, X.; Barouki, R.; Muller, M.J.; Wolffram, S.; Rimbach, G. Effect of quercetin on paraoxonase 1 activity - studies in cultured cells, mice and humans. *J. Physiol. Pharmacol.* **2010**, *61*, 99–105.
31. Fuhrman, B.; Aviram, M. Preservation of paraoxonase activity by wine flavonoids: Possible role in protection of LDL from lipid peroxidation. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 321–324, doi:10.1111/j.1749-6632.2002.tb02933.x.
32. Ustundag, B.; Bahcecioglu, I.H.; Sahin, K.; Duzgun, S.; Koca, S.; Gulcu, F.; Ozercan, I.H. Protective effect of soy isoflavones and activity levels of plasma paraoxonase and arylesterase in the experimental nonalcoholic steatohepatitis model. *Dig. Dis. Sci.* **2007**, *52*, 2006–2014, doi:10.1007/s10620-006-9251–9.
33. Gouedard, C.; Barouki, R.; Morel, Y.; Gouédard, C.; Barouki, R.; Morel, Y. Induction of the paraoxonase-1 gene expression by resveratrol. *Arter. Thomb. Vasc. Biol.* **2004**, *24*, 2378–2383, doi:10.1161/01.atv.0000146530.24736.ce.
34. Gupta, N.; Kandimalla, R.; Priyanka, K.; Singh, G.; Gill, K.D.; Singh, S. Effect of resveratrol and nicotine on PON1 gene expression: In vitro study. *Indian, J. Clin. Biochem.* **2014**, *29*, 69–73, doi:10.1007/s12291-013-0300–9.
35. Gouedard, C.; Barouki, R.; Morel, Y.; Gouédard, C.; Barouki, R.; Morel, Y.; Gouedard, C.; Barouki, R.; Morel, Y. Dietary polyphenols increase paraoxonase 1 gene expression by an aryl hydrocarbon receptor-dependent mechanism. *Mol. Cell Biol.* **2004**, *24*, 5209–5222, doi:10.1128/mcb.24.12.5209-5222.2004.
36. Krga, I.; Monfoulet, L.E.; Konic-Ristic, A.; Mercier, S.; Glibetic, M.; Morand, C.; Milenkovic, D. Anthocyanins and their gut metabolites reduce the adhesion of monocyte to TNFalpha-activated

endothelial cells at physiologically relevant concentrations. *Arch. Biochem Biophys* **2016**, *599*, 51–59, doi:10.1016/j.abb.2016.02.006.

37. Czank, C.; Cassidy, A.; Zhang, Q.; Morrison, D.J.; Preston, T.; Kroon, P.A.; Botting, N.P.; Kay, C.D. Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: A 13C-tracer study. *Am. J. Clin. Nutr.* **2013**, *97*, 995–1003, doi:10.3945/ajcn.112.049247.
38. De Ferrars, R.M.; Czank, C.; Zhang, Q.; Botting, N.P.; Kroon, P.A.; Cassidy, A.; Kay, C.D. The pharmacokinetics of anthocyanins and their metabolites in humans. *Br. J. Pharmacol.* **2014**, *171*, 3268–3282, doi:10.1111/bph.12676.
39. Schader, C.; Schiborr, C.; Frank, J.; Rimbach, G. Curcumin induces paraoxonase 1 in cultured hepatocytes in vitro but not in mouse liver in vivo. *Br. J. Nutr.* **2011**, *105*, 167–170, doi:10.1017/S0007114510004356.
40. Van Rymenant, E.; Grootaert, C.; Beerens, K.; Needs, P.W.; Kroon, P.A.; Kerimi, A.; Williamson, G.; García-Villalba, R.; González-Sarrías, A.; Tomas-Barberan, F.; et al. Vasorelaxant activity of twenty-one physiologically relevant (poly)phenolic metabolites on isolated mouse arteries. *Food Funct.* **2017**, *8*, 4331–4335, doi:10.1039/c7fo01273j.
41. Jaichander, P.; Selvarajan, K.; Garelnabi, M.; Parthasarathy, S. Induction of paraoxonase 1 and apolipoprotein A-I gene expression by aspirin. *J. Lipid Res.* **2008**, *49*, 2142–2148, doi:10.1194/jlr.m800082-jlr200.
42. Tvarijonaviciute, A.; Tecles, F.; Caldin, M.; Tasca, S.; Cerón, J. Validation of spectrophotometric assays for serum paraoxonase type-1 measurement in dogs. *Am. J. Vet. Res.* **2012**, *73*, 34–41, doi:10.2460/ajvr.73.1.34.
43. Browne, R.W.; Koury, S.T.; Marion, S.; Wilding, G.; Muti, P.; Trevisan, M. Accuracy and biological variation of human serum paraoxonase 1 activity and polymorphism (Q192R) by kinetic enzyme assay. *Clin. Chem.* **2007**, *53*, 310–317, doi:10.1373/clinchem.2006.074559.
44. Martinelli, N.; Girelli, D.; Olivieri, O.; Guarini, P.; Bassi, A.; Trabetti, E.; Friso, S.; Pizzolo, F.; Bozzini, C.; Tenuti, I.; et al. Novel serum paraoxonase activity assays are associated with coronary artery disease. *Clin. Chem. Lab. Med.* **2009**, *47*, 432–440, doi:10.1515/cclm.2009.108.
45. Gaidukov, L.; Tawfik, D.S. The development of human sera tests for HDL-bound serum PON1 and its lipolactonase activity. *J. Lipid Res.* **2007**, *48*, 1637–1646, doi:10.1194/jlr.D600045-JLR200.
46. Ferre, N.; Feliu, A.; Garcia-Heredia, A.; Marsillach, J.; Paris, N.; Ferré, N.; Feliu, A.; García-Heredia, A.; Marsillach, J.; París, N.; et al. Impaired paraoxonase-1 status in obese children. Relationships with insulin resistance and metabolic syndrome. *Clin. Biochem.* **2013**, *46*, 1830–1836, doi:10.1016/j.clinbiochem.2013.08.020.
47. Khersonsky, O.; Tawfik, D.S. Chomogenic and fluorogenic assays for the lactonase activity of serum paraoxonases. *Chem. Bio. Chem.* **2006**, *7*, 49–53, doi:10.1002/cbic.200500334.
48. Esatbeyoglu, T.; Ulbrich, K.; Rehberg, C.; Rohn, S.; Rimbach, G. Thermal stability, antioxidant, and anti-inflammatory activity of curcumin and its degradation product 4-vinyl guaiacol. *Food Funct.* **2015**, *6*, 887–893, doi:10.1039/c4fo00790e.
49. Wallace, T.C.; Slavin, M.; Frankenfeld, C.L. Systematic review of anthocyanins and markers of cardiovascular dise. *Nutrients* **2016**, *8*, 32, doi:10.3390/nu8010032.
50. Cassidy, A.; Bertoia, M.; Chiuve, S.; Flint, A.; Forman, J.; Rimm, E.B. Habitual intake of anthocyanins and flavanones and risk of cardiovascular disease in men. *Am. J. Clin. Nutr.* **2016**, *104*, 587–594, doi:10.3945/ajcn.116.133132.
51. Blesso, C.N. Dietary Anthocyanins and Human Health. *Nutrients* **2019**, *11*, 2107.
52. Kolehmainen, M.; Mykkänen, O.; Kirjavainen, P.V.; Leppänen, T.; Moilanen, E.; Adriaens, M.; Laaksonen, D.E.; Hallikainen, M.; Puupponen-Pimiä, R.; Pulkkinen, L.; et al. Bilberries reduce low-grade inflammation in individuals with features of metabolic syndrome. *Mol. Nutr. Food Res.* **2012**, *56*, 1501–1510, doi:10.1002/mnfr.201200195.
53. Karlsen, A.; Paur, I.; Bohn, S.K.; Sakhi, A.K.; Borge, G.I.; Serafini, M.; Erlund, I.; Laake, P.; Tonstad, S.; Blomhoff, R. Bilberry juice modulates plasma concentration of NF-κB related inflammatory markers in subjects at increased risk of CVD. *Eur. J. Nutr.* **2010**, *49*, 345–355, doi:10.1007/s00394-010-0092–0.

54. Mueller, D.; Jung, K.; Winter, M.; Rogoll, D.; Melcher, R.; Richling, E. Human intervention study to investigate the intestinal accessibility and bioavailability of anthocyanins from bilberries. *Food Chem.* **2017**, *231*, 275–286, doi:10.1016/j.foodchem.2017.03.130.
55. Amin, H.P.; Czank, C.; Raheem, S.; Zhang, Q.; Botting, N.P.; Cassidy, A.; Kay, C.D. Anthocyanins and their physiologically relevant metabolites alter the expression of IL-6 and VCAM-1 in CD40L and oxidized LDL challenged vascular endothelial cells. *Mol. Nutr. Food Res.* **2015**, *59*, 1095–1106, doi:10.1002/mnfr.201400803.
56. Youdim, K.A.; Martin, A.; Joseph, J.A. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radic. Biol. Med.* **2000**, *29*, 51–60, doi:10.1016/S0891-5849(00)00329–4.
57. Khateeb, J.; Gantman, A.; Kreitenberg, A.J.; Aviram, M.; Fuhrman, B. Paraoxonase 1 (PON1) expression in hepatocytes is upregulated by pomegranate polyphenols: A role for PPAR-gamma pathway. *Atherosclerosis* **2010**, *208*, 119–125, doi:10.1016/j.atherosclerosis.2009.08.051.
58. Rupérez, A.I.; López-Guarnido, O.; Gil, F.; Olza, J.; Gil-Campos, M.; Leis, R.; Tojo, R.; Cañete, R.; Gil, A.; Aguilera, C.M.; et al. Paraoxonase 1 activities and genetic variation in childhood obesity. *Br. J. Nutr.* **2013**, *110*, 1639–1647, doi:10.1017/s0007114513001967.
59. Rosenblat, M.; Gaidukov, L.; Khersonsky, O.; Vaya, J.; Oren, R.; Tawfik, D.S.; Aviram, M. The catalytic histidine dyad of high density lipoprotein-associated serum paraoxonase-1 (PON1) is essential for PON1-mediated inhibition of low density lipoprotein oxidation and stimulation of macrophage cholesterol efflux. *J. Biol. Chem.* **2006**, *281*, 7657–7665, doi:10.1074/jbc.M512595200.
60. Levy, D.; Reichert, C.O.; Bydlowski, S.P.; Levy, D.; Reichert, C.O.; Bydlowski, S.P. Paraoxonases activities and polymorphisms in elderly and old-age diseases: An overview. *Antioxidants* **2019**, *8*, 118, doi:10.3390/antiox8050118.
61. Phuntuwate, W.; Suthisisang, C.; Koanantakul, B.; Mackness, M.I.; Mackness, B. Paraoxonase 1 status in the Thai population. *J. Hum. Genet.* **2005**, *50*, 293–300, doi:10.1007/s10038-005-0255–7.
62. Atrahimovich, D.; Vaya, J.; Tavori, H.; Khatib, S. Glabridin protects paraoxonase 1 from linoleic acid hydroperoxide inhibition via specific interaction: A fluorescence-quenching study. *J. Agric. Food Chem.* **2012**, *60*, 3679–3685, doi:10.1021/jf2046009.
63. Deakin, S.; Leviev, I.; Gomaraschi, M.; Calabresi, L.; Franceschini, G.; James, R.W. Enzymatically active paraoxonase-1 is located at the external membrane of producing cells and released by a high affinity, saturable, desorption mechanism. *J. Biol. Chem.* **2002**, *277*, 4301–4308, doi:10.1074/jbc.M107440200.
64. Graves, T.L.; Scott, J.E. A High Thoughput Serum Paraoxonase Assay for Discovery of Small Molecule Modulators of PON1 Activity. *Curr. Chem. Genomics* **2008**, *2*, 51–61, doi:10.2174/1875397300802010051.
65. Latruffe, N.; Menzel, M.; Delmas, D.; Buchet, R.; Lançon, A. Compared Binding Properties between Resveratrol and Other Polyphenols to Plasmatic Albumin: Consequences for the Health Protecting Effect of Dietary Plant Microcomponents. *Molecules* **2014**, *19*, 17066–17077, doi:10.3390/molecules191117066.
66. Nozaki, A.; Hori, M.; Kimura, T.; Ito, H.; Hatano, T. Interaction of Polyphenols with Proteins: Binding of (−)-Epigallocatechin Gallate to Serum Albumin, Estimated by Induced Circular Dichoism. *Chem. Pharm. Bull. (Tokyo).* **2009**, *57*, 224–228, doi:10.1248/cpb.57.224.
67. Cao, H.; Liu, X.; Ulrih, N.P.; Sengupta, P.K.; Xiao, J. Plasma protein binding of dietary polyphenols to human serum albumin: A high performance affinity chomatography approach. *Food Chem.* **2019**, *270*, 257–263, doi:10.1016/j.foodchem.2018.07.111.
68. Kay, C.D.; Kroon, P.A.; Cassidy, A. The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products. *Mol. Nutr. Food Res.* **2009**, *53*, S92–S101, doi:10.1002/mnfr.200800461.
69. Federica Rizzi Elena Dogliotti, Annalisa Terranegra, Erika Salvi, Daniele Braga, Flavia Ricca, Sara Lupoli, Alessandra Mingione, Francesca Pivari, Caterina Brasacchio, Matteo Barcella, Martina Chittani, Francesca D'Avila, Maurizio Turiel, Monica Lazzaron, C.C.; Rizzi, F.; Conti, C.; Dogliotti, E.; Terranegra, A.; Salvi, E.; Braga, D.; Ricca, F.; Lupoli, S.; Mingione, A.; et al. Interaction between polyphenols intake and PON1 gene variants on markers of cardiovascular disease: A nutrigenetic observational study. *J. Transl Med.* **2016**, *14*, 10, doi:10.1186/s12967-016-0941–6.

70. Atkinson, T.J.; Halfon, M.S. Regulation of gene expression in the genomic context. *Comput. Struct. Biotechnol. J.* 2014, *9*, doi:10.5936/csbj.201401001.

nutrients

APPENDIX 8

An review:

Polyphenol Effects on Cholesterol Metabolism via Bile Acid Biosynthesis, CYP7A1: A Review

nutrients

MDPI

Review

Polyphenol Effects on Cholesterol Metabolism via Bile Acid Biosynthesis, CYP7A1: A Review

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Abstract: Atherosclerosis, the main contributor to coronary heart disease, is characterised by an accumulation of lipids such as cholesterol in the arterial wall. Reverse cholesterol transport (RCT) reduces cholesterol via its conversion into bile acids (BAs). During RCT in non-hepatic peripheral tissues, cholesterol is transferred to high-density lipoprotein (HDL) particles and returned to the liver for conversion into BAs predominantly via the rate-limiting enzyme, cholesterol 7 α-hydroxylase (CYP7A1). Numerous reports have described that polyphenol induced increases in BA excretion and corresponding reductions in total and LDL cholesterol in animal and in-vitro studies, but the process whereby this occurs has not been extensively reviewed. There are three main mechanisms by which BA excretion can be augmented: (1) increased expression of CYP7A1; (2) reduced expression of intestinal BA transporters; and (3) changes in the gut microbiota. Here we summarise the BA metabolic pathways focusing on CYP7A1, how its gene is regulated via transcription factors, diurnal rhythms, and microRNAs. Importantly, we will address the following questions: (1) Can polyphenols enhance BA secretion by modulating the CYP7A1 biosynthetic pathway? (2) Can polyphenols alter the BA pool via changes in the gut microbiota? (3) Which polyphenols are the most promising candidates for future research? We conclude that while in rodents some polyphenols induce CYP7A1 expression predominantly by the LXRα pathway, in human cells, this may occur through FXR, NF-KB, and ERK signalling. Additionally, gut microbiota is important for the de-conjugation and excretion of BAs. Puerarin, resveratrol, and quercetin are promising candidates for further research in this area.

Keywords: atherosclerosis; reverse cholesterol transport; diurnal rhythms; microRNA; ASBT; flavonoid; phenolic acid; catechin; anthocyanin

1. Introduction

Atherosclerosis, the main contributor to coronary heart disease, is characterised by an accumulation of lipids in the arterial wall [1]. Polyphenols have been shown to confer beneficial effects against cardio-metabolic diseases. Among the mechanisms proposed for their beneficial effects is the alteration of bile acid metabolism. Here, we give a comprehensive review of research on the molecular mechanisms through which polyphenols exert their beneficial effects focusing on CYP7A1 and bile acid metabolism. The key points raised in this review include:

1. Polyphenols have been shown to have a wide range of beneficial effects, of note red wine, rich in flavonoids, phenolic polymers and resveratrol are promising as possible targets for further investigation.
2. As well as giving a state-of- the-art review on the mechanisms through which polyphenols exert their beneficial effects focussing on CYP7A1 and bile acid metabolism, critical points that will be of benefit to clinical nutritionists, academic experts in the area of bioactive food compounds, and possible stakeholders have also been raised in this review namely;

    a. While polyphenols do have effects on bile acid metabolism, it should be born in mind that species differences, time of cull, dose, and length of treatment can also affect the results and as such, may lead to discrepancies between studies. As such, there is a need to standardise animal studies, ensuring that these factors are correctly reported.
    b. Polyphenols confer their beneficial effects partly through altering the microbiome, thus they could have useful prebiotic-like functions.
    c. There is a lack of data on the effects conferred by the parent compound versus those of their metabolic products, and future studies should aim to determine the effects of both the parent compounds and their metabolites, with particular emphasis on the gut microbiota.
    d. Further studies are required in humans to substantiate the mechanisms proposed using animal and cell culture models.

1.1. Reverse Cholesterol Transport

The reverse cholesterol transport (RCT) pathway removes excess cholesterol from the peripheral system including from lipid laden macrophages, thus preventing foam cell accumulation during atherosclerosis development. In this process, cholesterol is returned to the liver where it is converted into BAs predominantly via the cytochrome P450 enzyme, cholesterol 7 α-hydroxylase (CYP7A1), for subsequent excretion into the faeces (Figure 1).

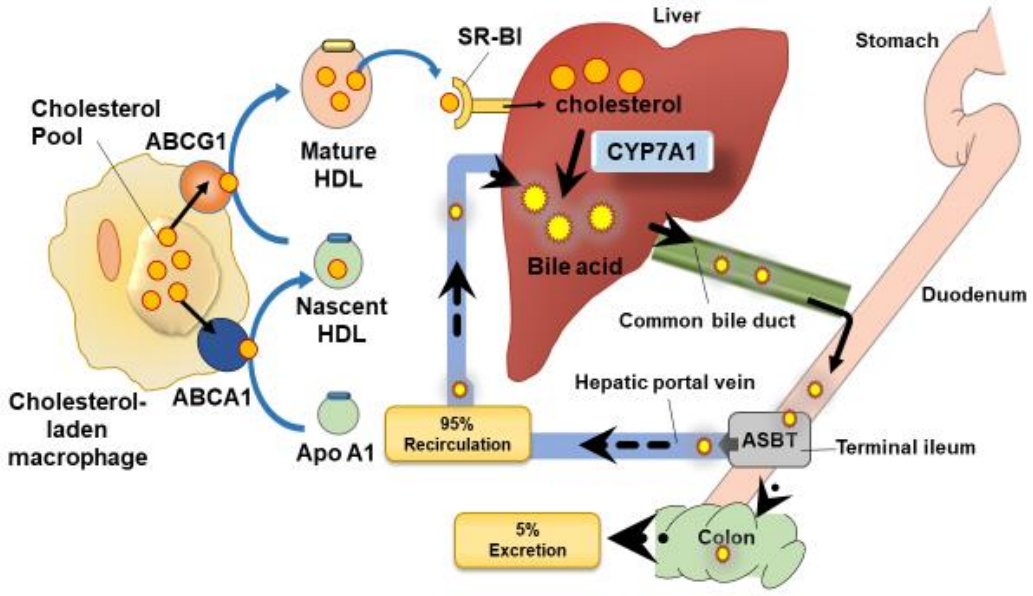

Figure 1. The enterohepatic re-circulation of bile acids via reverse cholesterol transport. Cholesterol laden macrophages in the arterial wall deliver cholesterol via the ABCA1 transporter to lipid free apoA-I, preventing foam cell formation and also forming nascent HDL particles. Further lipidation of the nascent HDLs occurs via ABCG1. Cholesterol is delivered to the liver from mature HDL particles via specific HDL cholesterol efflux (CE) uptake by a scavenger receptor class B type I (SR-BI). In the liver, cholesterol is converted into BAs predominantly by the CYP7A1 neutral (classic) pathway. The BAs travel via the bile duct to the intestine, where they are de-conjugated via the bacteria and excreted or re-circulated (95%), usually in their conjugated form via passive diffusion or via active transport via the apical sodium dependent BA transporter (ASBT).

In humans, approximately 500 mg/day of cholesterol is converted into bile acids (Bas) in the liver and lost in the faeces [2]. Two pathways, namely the neutral (classic) and the alternative (acidic) pathways, are involved in cholesterol metabolism, with CYP7A1 being the rate-limiting enzyme in the former, and CYP7A1 being responsible for cholesterol metabolism in the latter. Both pathways result in the formation of the primary BAs chenodeoxycholic acid (CDCA) and cholic acid (CA), the neutral pathway being the most predominant [3]. The acidic pathway only contributes 9% and 25% of total BAs in humans and mice, respectively [4]. The biochemistry of BA biosynthesis is reviewed elsewhere [2,5] and is summarised in Figure 2.

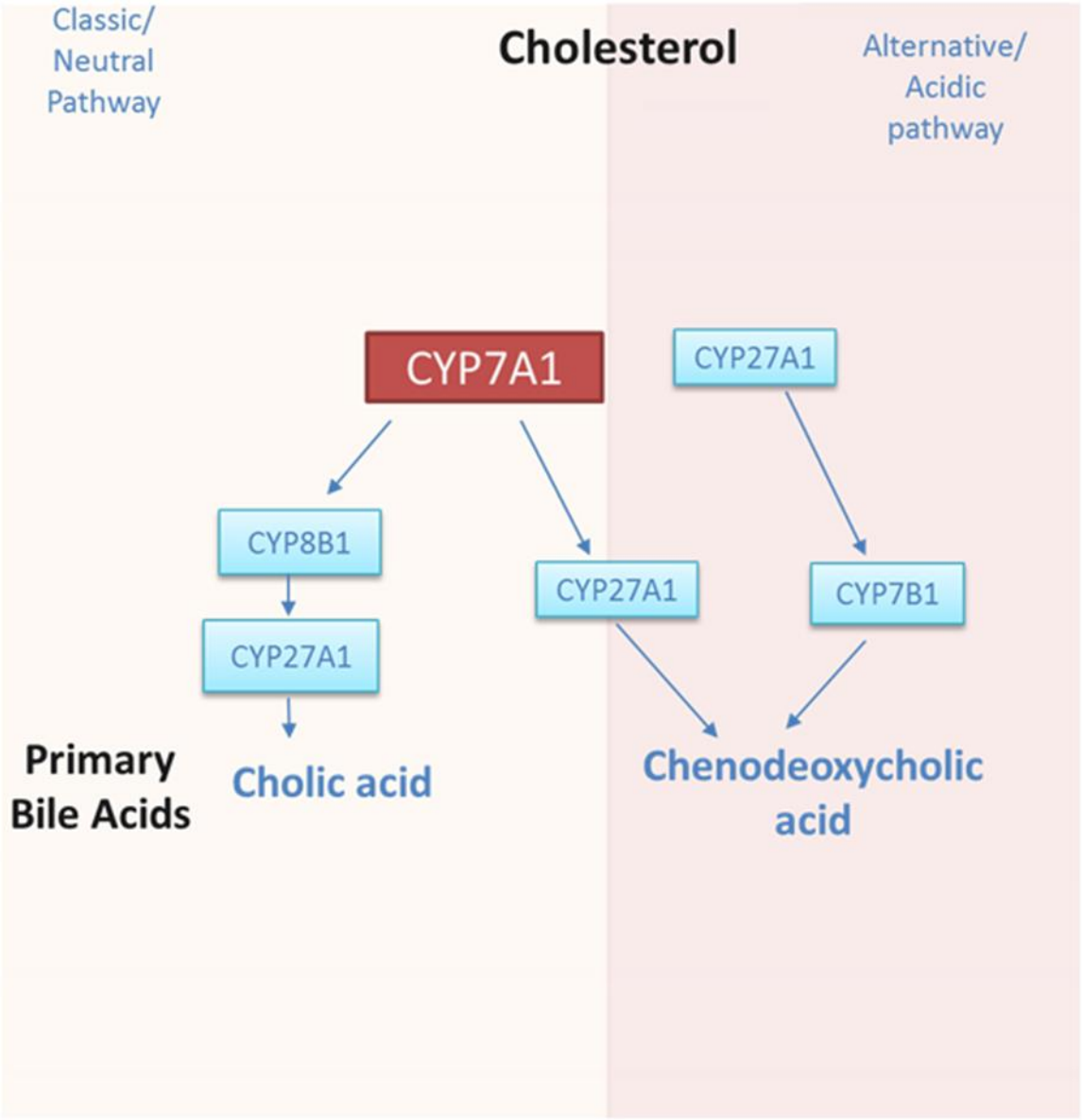

Figure 2. A summary of the main enzymes involved in the classic and alternative bile acid biosynthesis pathways. The classic pathway is controlled by the rate limiting enzyme CYP7A1 and the alternative pathway is controlled by CYP27A1; both pathways culminate in the production of cholic acid (CA) and chenodexoycholic acid (CDCA), the ratio of which depends on the activity of CYP8B1. Briefly, bile-acid biosynthesis begins with the modification of the ring structure of cholesterol, which involves oxidation and shortening of the side chain [2]. In the classic pathway, cholesterol is converted into 7 α-hydroxycholesterol by CYP7A1 and in subsequent steps, cytochrome P450 Family 8 Subfamily B Member 1a (CYP8B1) and sterol 27-hydroxylase (CYP27A1) are required for the synthesis of cholic acid (CA). Without CYP8B1, the product is chenodeoxycholic acid (CDCA), which is formed via the activity of CYP27A1 alone. The acidic pathway (or alternative pathway) is initiated by CYP27A1 and relies on 25-hydroxycholesterol 7-alpha-hydroxylase (CYP7B1) to produce CDCA.

Before excretion into bile, primary BAs are conjugated with amino acids glycine and to a lesser degree taurine in humans [6]. In rodents, taurine is almost exclusively used for conjugation [6]. This increases solubility, minimises passive absorption, and makes the BAs resistant to cleavage by pancreatic carboxypeptidase. BAs are secreted from the gallbladder via the bile duct into the intestine and can be metabolised into their respective secondary BAs

via the gut microbiota, which are also responsible for de-conjugation. Conjugated BAs are actively exported from the liver via the bile salt export pump (BSEP)/ABCB11, which in humans preferentially transports conjugated BAs, but can also transport unconjugated BAs [7]. The main function of BAs is to facilitate digestion in the gut and their synthesis inadvertently regulates lipid concentrations. A list of all known BAs and their abbreviations is given in Supplementary Table S1. BAs are reabsorbed in the intestine by either passive diffusion or by active transport, which occurs via the apical sodium BA transporter (ASBT) in the terminal portion of the ileum [8]. Once reabsorbed, BAs travel through the hepatic portal vein and are returned to the liver via specific transporters, with approximately 95% of BAs being recirculated back to the gallbladder in a process termed enterohepatic circulation, and the rest is excreted in the faeces (Figure 1). Typically, cholesterol synthesis equals its secretion as BAs; however, this equilibrium can be disturbed during disease states or by diet [9].

1.2. Regulation of CYP7A1 *by Dietary Cholesterol, Circadian Rhythm, Transcription Factors, and microRNAs*

Diet can regulate BA synthesis, particularly the intake of a cholesterol rich diet. In wild-type mice, feeding a high cholesterol diet stimulated BA synthesis by activating LXRα to induce *CYP7A1* gene transcription [10–12]. Cholesterol treatment of HepG2 cells also moderately induces *CYP7A1* gene expression [13]. In contrast, another study using HepG2 cells, cholesterol dose dependently decreased *CYP7A1* expression [14], which is supported by a mouse study [15]. Although it has been suggested that mice have a different response to cholesterol when compared to humans, here, comparable effects were observed on BA pool size, faecal BA excretion, and plasma cholesterol levels between humans, transgenic mice, and wild-type CYP7A1 mice. This suggests that another mechanism other than LXR signalling alters BAs on a high cholesterol diet, perhaps the microbiota [16]. There are also differences between rodents and humans, for example LXRα-CYP7A1 signalling, is redundant in humans due to the lack of LXRE in the human *CYP7A1* gene promoter, but this pathway is well known in rodent models. Nevertheless, FXR signalling is homologous between mice and humans and is a well-studied mechanism of *CYP7A1* control. It is logical that cholesterol, the substrate for *CYP7A1*, would increase CYP7A1 levels, but the data published to date are equivocal and may depend on the presence of a functional LXRα receptor.

One key mechanism is the circadian regulation of CYP7A1. *Cyp7a1* levels in mice have been shown to peak mid-morning to noon, although this is also influenced by the fed and non-fed state [17]. This is particularly problematic when animals treated with different diets are culled at different times over the day as this would dramatically alter the results and lead to erroneous interpretation [17]. The circadian control of *Cyp7a1*, along with other clock associated genes, has also been shown to parallel changes in triglyceride and total cholesterol [18]. A summary of circadian genes involved in the regulation of *Cyp7a1* is shown in Table 1. *Cyp7a1* is also regulated by microRNAs, which are non-coding RNAs of 15–25 bases that bind to complementary sequences in the 3′-UTR regions of target mRNA to repress translation. The effects and targets of microRNAs on *Cyp7a1* and cholesterol metabolism are also summarised in Table 1. The concept that diet can regulate BA synthesis is not a new one, in fact fibre is well known to regulate BA synthesis [19–23], however, the idea that bio-actives within food, particularly polyphenols, can increase CYP7A1 without increased food consumption is an exciting field and will be considered in the following sections.

Table 1. A summary of the transcription factors and microRNAs involved in Cyp7a1 gene regulation in humans and rodents.

Factor	Humans/Human Cell Lines	Rodents	Ref.
Farnesoid X receptor, retinoic acid receptor and small heterodimer partner (FXR-RXR and SHP)	FXR is highly expressed in both the liver and ileum tissue. Ligand binding to FXR allows translocation from the cytoplasm to the nucleus to bind RXR at FXR-response elements. FXR is not able to bind to *CYP7A1* but can bind to the promoter of the *SHP* gene. SHP represses *CYP7A1* gene expression by binding to human α-fetoprotein transcription factor (FTF). FXR-RXR complex also binds illeal FGF19 (humans) or FGFR4 (mice) translocates to the liver, activates FGR4 which inhibits *c CYP7A1* transcription.	The same as humans, however, FTF is called liver receptor homolog-1 (LRH-1) in mice.	[24–26]
Pregnane X receptor (PXR)	PXR activation by specific bile acids such as lithocholic acid (LCA) leads to the repression of bile acid synthesis by binding and inactivating the transcription factor, hepatocyte nuclear factor 4 alpha (HNF4α) so that it can no-longer bind to its transcriptional co-activator, proliferator-activated receptor γ co-activator 1-α (PGC1α) to induce *CYP7A1* transcription.		[27]
Liver X receptor α (LXRα)	LXRα cannot bind to the human *CYP7A1* promoter due to an alteration of the DR4 motif in the BARE-I sequence. Therefore, in humans LXRα does not play a role in the regulation of CYP7A1 gene expression.	Unlike in humans, LXRα can directly bind to the *Cyp7a1* promoter to upregulate expression.	[28]
Hepatocyte nuclear factor 4 alpha/Peroxisome proliferator-activated receptor γ co-activator 1-α (HNF4α /PGC1α)	HNF4α is a transcription factor that upregulates *CYP7A1* by directly binding to its promoter along with the *trans*-activator PGC-1α.	Same as humans.	[29–32]
Peroxisome Proliferator Activated Receptor Alpha (PPARα)	In vitro PPARα over-expression in human liver cells has been shown to reduce *CYP7A1*gene expression. However, when activators of PPARα where added to non-over-expressing cells a moderate amount of inhibition was observed.	PPARα knock out mice did not show altered *Cyp7a1* levels.	[31,33]
Peroxisome Proliferator Activated Receptor gamma (PPARγ)	PPARγ activation induced *CYP7A1* expression in HepG2 cells		[34]

Forkhead box protein O1 (FoxO1)	FoxO1 is an in-direct suppressor of *CYP7A1*, there is no binding site for FoxO1 on the human *CYP7A11* promoter. FoxO1 inhibits *CYP7A1* by inhibiting expression of HNF-4α and PGC-1α TFs.	FoxO1 has the opposite function in mice and upregulates *Cyp7a1* directly by binding to its promoter.	[35,36]
Nuclear receptor subfamily 1, group D, member 1 (NR1D1 or Rev-Erba)	NR	Competes for the promoter of the clock gene *Bmal1* and mediate the circadian regulation of *Cyp7a1*	[37,38].
Per1 and *Per2*	NR	Genetic ablation in mice disrupts normal BA control, increases serum BA and in parallel reduces *dbp* and *Cyp7a1* expression in both rats and mice	[39,40].
D site albumin promoter binding protein (DBP)	Gain-of-function studies have shown that DBP serves as a circadian activator of *CYP7A1* transcription.	*Cyp7a1* peaks after dark	[41–43]
Enhancer binding protein C/EBPβ-LAP	Binds cyp7a1 promoter site at DBP		[39]
miR-33		Located in the intron sequence of SREBP and regulates *Cyp7a1* expression possibly synergistically to control hepatic cholesterol metabolism and BA synthesis	[44–47]
miR-144-3p and miR-99a-3p	Target *CYP7A1* and other non-alcoholic fatty liver disease related genes		[44]
miR-122 and miR-422	Cyp7a1 also has recognition sequences for miR-122 and miR-422 in its 3′-UTR. A synthetic miR-122 mimic inhibits *CYP7A1*expression in vitro and miR-122 inhibition has shown the opposite effect		[45]
miR-24 and miR-34	Indirectly decrease *CYP7A1* by decreasing HNF4α transcription factor		[48]

miR-17	Leads a reduction in CYP7A1 mRNA expression	Reduces *Cyp7a1* mRNA expression accompanied by hepatic steatosis	[30].

2. Current Status of Knowledge: Polyphenols That Regulate Bile Acid Synthesis and CYP7A1

Numerous studies have assessed the effects of polyphenols on BA excretion and CYP7A1 expression and the mechanisms by which they alter BA excretion and CYP7A1expression have been summarised in Supplementary Table S2; these were mostly obtained from rodent studies showing a depletion of hepatic cholesterol when fed polyphenols, which in some instances is linked to an increase in CYP7A1 activity and LXRα signalling. Polyphenols are subdivided into different categories: flavonoids, isoflavonoids, lignans, stilbenes, phenolic acids, and phenolic polymers (Supplementary Figure S1). It is worth noting that all types of polyphenols are only partially bioavailable and subject to both phase-2 conjugation and catabolism by the gut microbiota, and the reader is referred to some excellent reviews of the bioavailability and metabolism of polyphenols and how these processes can affect their biological activities [49–53]. Currently, evidence for whether the known effects of polyphenols on BA metabolism and CYP7A1 are mediated by the parent polyphenols or their metabolites, or a combination of both is limited; this is an important area for future research. There are multiple pathways through which polyphenols confer their beneficial effects including those involved in inflammation. These have been substantially reviewed elsewhere [54,55] Another biological activity of polyphenols is as antioxidant molecules that have the potential to mop up free radicals. Although this has been somewhat challenging to prove unequivocally in humans, it is notable that there is a significant body of evidence to show that consumption of specific polyphenols such as those in olive oil can effectively reduce the levels of oxidised LDL [56]. In the following sections, we review the effects of each sub-group of polyphenols on CYP7A1 expression, BA metabolism/excretion, and comment on the mechanism of action. We then further discuss the emerging research that polyphenols can mediate cholesterol efflux and microbial changes in the gut, subsequently modulating bile acid excretion.

2.1. Flavonoids

Flavonoids are water soluble polyphenolic molecules that consist of six major subgroups: flavanols (catechins), anthocyanins, flavonols, flavanones, and flavones (see Supplementary Figure S1). The effects of flavonoids on the HDL delivery of cholesterol to the liver via RCT have been recently reviewed [57], however, the effects of polyphenols on bile acid biosynthesis and efflux was not described.

2.1.1. Flavanols

Flavanol-rich green tea has been shown to increase BA excretion, however, the link to CYP7A1 is tentative. For example, Lung Chen tea has been shown to reduce serum cholesterol and increase faecal BA excretion in hypercholesteraemic rats, without altering Cyp7a1 enzyme activity [58]. *Cyp7a1* gene expression was not changed in C57BL mice fed green tea extract (GTE) over six weeks [59]. Despite this, there was an increase in circulating BAs and excreted faecal cholesterol, although this was only apparent once normalised to the amount of faeces excreted per day as the mice on the GTE produced more faeces overall [15]. Interestingly, in the same study, GTE dramatically increased *Cyp27a1* mRNA expression in mice fed a high cholesterol diet, but not in mice fed a normal chow diet; indicating a shift from the neutral pathway to the acidic pathway only in the presence of high cholesterol [15]. Several studies have shown an increase in *Cyp7a1* expression in response to catechin treatment. For example, in rats, epigallocatechin gallate (EGCG) decreased bile acid-independent bile flow but not excretion, whilst increasing *Cyp7a1* mRNA expression and increasing circulating BAs [60]. EGCG increased Cyp7a1 protein levels in C57BL/6 mouse livers, however, BAs were not

measured [61]. Finally, an in vitro study using HepG2 cells has shown that catechins increase *CYP7A1*mRNA levels with epicatechin gallate showing the greatest fold induction [62]. In contrast, EGCG is also known to be an activator of FXR signalling [63], which would in turn supress *CYP7A1*expression. Studies in our lab confirm a reduction of *CYP7A1* gene expression in human liver HepG2 cells treated with 5 μM EGCG in the absence of serum, which is consistent with the hypothesis that EGCG is an activator of farnesoid X receptor (FXR) signalling (unpublished data). Interestingly, in the presence of chenodeoxycholic acid, another FXR inhibitor, EGCG acts as a suppressor of FXR signalling in vitro [63]. *CYP7A1* levels were not measured, but it would be fascinating to explore the role of EGCG on CYP7A1expression in the presence and absence of FXR inhibitors.

2.1.2. Anthocyanins

There are very limited data on the effects of anthocyanins on BAs. In one study, red pericarp glutinous rice, rich in anthocyanins, induced *Cyp7a1* expression when fed to hypercholesterolemic C57BL/6 mice when compared to brown rice [64]. A decrease in hepatic cholesterol was also observed although faecal cholesterol or BAs were not measured [64]. Lingonberries, which contain high levels of anthocyanins, have been shown to increase *Cyp7a1* expression and decrease atherosclerotic plaque and triglyceride concentration in *ApoE-/-* mice on a high fat diet [65]. The relative faecal abundance of the bacterial genera *Bacteroides*, *Parabacteroides*, and *Clostridium* were also increased [65]. In type-2 diabetic mice, Buckwheat sprouts, which contain cyanidin 3-O-glucoside (C-3-G) [66] and C-3-G alone, increased faecal BAs and *Cyp7a1* mRNA expression in the liver whilst correspondingly decreasing serum cholesterol, liver cholesterol, and triglycerides and consequently reduced atherosclerosis [67,68]. However, buckwheat protein has also been investigated as the active cholesterol lowering component [69]. High doses of polyphenol-rich Lonicera caerulea berry extract, which contains mainly anthocyanins, significantly upregulated *Cyp7a1* gene expression, reduced the expression of SREBP-1C, SREBP2, miR-33, and miR-122 and caused a reduction in cholesterol, LDL, and triglyceride in Sprague Dawley rats [70]. To our knowledge, all the published anthocyanin data that have examined changes in BA levels or *Cyp7a1* expression have utilised mouse models. However, human cells may respond differently, as in mouse primary hepatocytes, C-3-G treatment increased *Cyp7a1* expression in an LXRα dependent way [68]. Indeed, using HepG2 cells, we showed that C-3-G does not increase *CYP7A1* mRNA expression (data unpublished), perhaps because human cells do not possess functional LXRα.

2.1.3. Flavonols

Quercetin, a flavonol found in capers, has been shown to elevate hepatic Cyp7a1 as well as LXRα at both mRNA and protein levels in male Wistar rats along with increased secretion of BAs and total hepatic BAs [71]. Importantly, quercetin also increased the expression of hepatic ATP binding cassette transporter G1 (ABCG1) mRNA and protein expression, indicating that quercetin may be involved in the regulation of hepatic cholesterol efflux [71]. Black bean seed coat extract predominantly containing quercetin 3-O-glucoside significantly stimulated the expression of Cyp7a1 protein in the liver and faecal BAs in C57BL mice [72]. A combination of quercetin and leucodelphinidin or quercetin with Banyan tree, derived leucopelargonin and leucocyanin significantly increased hepatic and faecal BAs in hypercholesteraemic rats and correspondingly reduced L-LDL cholesterol with the former also increasing HDL [73,74]. Overall, there is some evidence from animal studies that quercetin alone or in combination with other flavonoids can induce BA excretion via induction of CYP7A1. Other flavonols such as kaempferol have been shown to increase hepatic CYP7A1, faecal cholesterol, and BAs through mechanisms that may involve its binding to LXRα [75].

Human interventions and in vitro studies are still required to examine the role of quercetin and kaempferol on Cyp7a1 and lipid metabolism.

2.1.4. Flavanones, Flavones, and Isoflavones

Naringin (a flavanone) occurs naturally in citrus fruits, especially in grapefruit, where it is responsible for the fruit's bitter taste. Naringin, has been shown to induce LDL-receptor and *CYP7A1* expression in HepG2 cells through the NF-κB and ERK signalling pathway as well as through PPARy, which occurs in a dose-dependent manor [76,77]. Its aglycone naringenin has been shown to be a partial agonist of LXRα in cells transfected with a reporter construct [76]. In contrast, using computer modelling of tetrahydro-flavanones (cryptochinones A–D), it was shown that flavanones may behave as FXR agonists to decrease *CYP7A1* mRNA expression [78]. The only one study that investigated the effects of flavones from a leaf extract of Xanthosoma sagittifolium showed no effects on total Bas, but reduced secondary BAs in rats [79,80].

When administered to mice and rats, an isoflavonoid puerarin (from arrowroot) increases hepatic *Cyp7a1* expression and suppresses serum and hepatic cholesterol, although in HepG2 cells, no changes were observed [81–83]. Additionally, Xuezhikang, an isoflavone-rich extract of red-yeast-rice and *Erythrina lysistemon* also rich in isoflavones, increased hepatic *Cyp7a1* expression and BA excretion in high-fat fed mice and in ovariectomised rats, respectively [84,85]. The bacterial isoflavone metabolite equol has also been shown to alter BA metabolism by increasing hepatic *CYP7A1* mRNA levels in the chicken embryos liver [86]. Soymilk and fermented soymilk, which contain both isoflavones, bioactive proteins, and peptides, have also been shown to attenuate hepatic cholesterol and triglycerides levels and increase hepatic *Cyp7a1* gene expression in Sprague Dawley rats [87]. It is not clear whether the effects can be attributed to either the flavonoids or the proteins/peptides or both, as soy protein alongside a high-fat diet also increased hepatic *Cyp7a1* mRNA in male Syrian Golden hamsters [88]. In addition, soy protein isolate has been shown to induce *Cyp7a1* hepatic expression and reduce hepatic cholesterol in rats [89]. The only study in humans showed no effects [83].

2.2. Stillbenes

The anti-atherosclerotic effects of resveratrol (3,5,4′-trihydroxy-trans-stilbene), which is found in the skin of grapes, blueberries, raspberries, and mulberries, have been widely studied in mouse models and human cell lines [90]. In mice on a high fat diet and in HepG2 cells, resveratrol increased CYP7A1 mRNA expression with activity being increased in the former [91]. Similar results were observed in HepG2 cells treated with resveratrol or resveratrol-glucuronides along with a concurrent decrease in cholesterol content and an increase in bile salt export protein (BSEP), respectively [92]. In alpha-Naphthylisothiocyanate (ANIT) induced liver injury in rats, resveratrol restored FXR and *Cyp7a1* expression and BA secretion [93]. Donryu rats implanted with an ascites hepatoma cell line and given resveratrol showed a dose-dependent reduction in serum cholesterol and excreted BAs [94]. Thus, supporting the evidence that resveratrol or its metabolites (aglycone and glucuronide), which are bioavailable in bile and plasma, 4 to 8 h after administration, may be functional bio-actives [95]. Resveratrol may confer its beneficial effects through FXR signalling targeting SIRT1, which acetylates FXR and prevents its binding with RXRα, consequently inhibiting its binding and activation of the *Cyp7a1* repressor Shp [96,97]. Resveratrol has also been shown to alter the gut microbiota profile in C57BL/6J and *ApoE-/-* mice as well as bile salt hydrolase activity and de-conjugation, thus increasing faecal excretion. This was associated with the repression of enterohepatic FXR and FGF15 signalling and increased *Cyp7a1* expression and hepatic BA synthesis [98]. In

antibiotic treated mice, resveratrol has no such effects, indicating the importance of gut microbiota in resveratrol mediated effects [98].

2.3. Phenolic Acids and Phenolic Polypmers

Phenolic acids such as vanillic, caffeic, ferulic, and gallic acid are found in high concentrations in berries, tea, whole grains, and wine. A fermented Chinese tea, which contains a range of phenolic acids including catechin and gallic acid, has been shown to reduce LDL cholesterol in a 3-month double-blind randomised study of health and hypercholesterolemic patients, although BA levels were not measured [99–101]. Similarly, in hypercholesteraemic rats, an 8-week administration of the tea increased faecal BAs, but not *Cyp7a1* expression [58]. A separate study showed decreases in serum LDL and total cholesterol in hypercholesteraemic rats after 3-weeks of feeding [102]. Indeed, a phenolic acid, chlorogenic acid, found in green tea, increased in *Cyp7a1* mRNA expression in 129/Sv mice, indicating that at least some of the phenolic acid components are bioactive [103].

The beneficial effects of some phenolic polymers found in raisins, grapes, and wine (high in fibre but also tannins (proanthocyanidins)) in humans are however questionable as two human studies from the same lab showed that consuming 80–120 g raisins over a two to nine week period decreased total faecal BAs, and in another study, when tartaric acid was fed to participants, no effects on BAs were observed [104–106]. In contrast, 2% tannin polymers from raisins significantly increased faecal BAs and lowered LDL cholesterol in mice, although tannin monomers did not have any effect [107]. Using an in vitro study, Camire et al. proposed that the fibre in chopped raisins act as a BA sequestrant to prevent enterohepatic re-circulation, although the role of polymeric tannins cannot be ruled out as gallic acid does directly bind to taurocholic, taurodeoxycholic, and glycodeoxycholic BAs in vitro, and ellagic acid has been shown to induce genes involved in BA synthesis in mice [108–110]. It is therefore evident that some raisin constituents may promote BA secretion in rodents, tannin polymers (but not monomers) being among them, although their effects in humans is yet to be assessed.

2.4: Grape Juice, Wine and Grape Seed Extract

In-vivo and vitro effects of grapes, wine, and grape seed extract (GPSE) on BA secretion have recently been reviewed with moderate increase in *Cyp7a1* after GPSE treatments of hamsters on a 0.1% cholesterol diet while red wine procyanidins induced hepatic *Cyp7a1* in Wistar rats along with reduced LDL-C [111–113]. Grape juice fed to rats also increased primary BA but reduced secondary BA in the intestinal contents. Interestingly faecal counts of *Lactobacillus* and *Bifdobaceterium* were also increased [114]. In FXR knockout mice, GPSE induced faecal BA output and downregulated genes involved in intestinal BA absorption and transport. This correlated with increased *Cyp7a1* mRNA expression in the liver, decreased circulating LDL cholesterol, and decreased intestinal *Fgf15* expression [115]. In contrast, fisetin a flavonol from red wine, decreased *Cyp7a1* mRNA expression in Sprague Dawley rats, when compared to controls of mice fed a high fat diet [116]. Despite this, the rats still exhibited decreased plasma total cholesterol and LDL-cholesterol, along with decreased hepatic cholesterol content [116]. Perhaps the alternative pathway regulated by CYP27A1 may be responsible for altered BA secretion in this instance. Procyanidins may also have long-term effects; for example, in the alteration of reverse cholesterol transport in the adult offspring after intake of grape procyanidins during gestation and lactation [117]. Overall, the evidence suggests GPSE induces *Cyp7a1* mRNA expression and BA secretion into faeces. There is less evidence for wine and grape juice, although the existing publications support the same trend presented with GPSE. Once again, studies in humans are lacking.

3. Polyphenol Mediated Mechanisms of Action

Understanding the mechanisms through which polyphenols modulate CYP7A1 to regulate cholesterol and bile acid metabolism is important and this review has highlighted that multiple pathways may be involved. These include regulation through the NF-Kβ/ERK and SIRT-RXR-FXR (LXR) signalling pathways, modification of circadian rhythm associated genes, reverse cholesterol transport, and bile salt hydrolyses (Figure 3). While there is limited experimental data on the effects of polyphenols on microRNAs and CYP7A1expression, using a database that predicts the target for microRNAs in messenger RNA in rats, several microRNAs have been recognised to target CYP7A1, and some of these microRNAs have been shown to be modulated by polyphenol supplementation (Figure 3). Polyphenols have also been shown to regulate intestinal BA transporters and alter the gut microbial composition (Figure 3) to regulate the excretion of BAs as will be discussed in Sections 3.1 and 3.2.

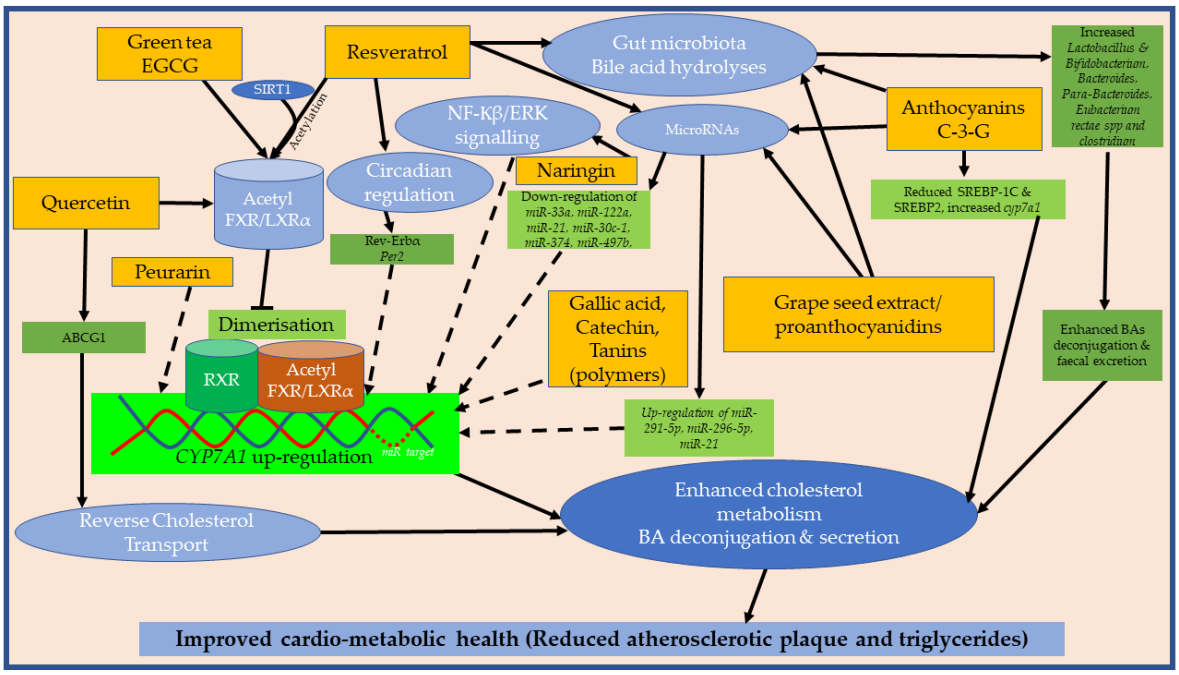

Figure 3. Putative mechanisms through which polyphenols modulate CYP7A1 to promote cholesterol metabolism and bile acid deconjugation, consequently attenuating atherosclerosis plaque development. Resveratrol, epigallocatechin gallate (EGCG), and quercetin increase *cyp7a1* by employing SIRT1 to acetylate FXR/LXRα; preventing its dimerisation with RXR, which then inhibits its binding and activation of CYP7A1 repressor [77,93]. Resveratrol can activate circadian proteins [118,119] and genes that subsequently upregulate *cyp7a1* and together with C-3-G, procyanidins, and naringin, can downregulate or upregulate certain microRNAs to promote CYP7A1 expression [45,120–123]. Alternatively, these may also increase bile acid hydrolases and certain gut microbiota species involved in the deconjugation and excretion of bile acids [124–126]. Dashed lines indicate mechanisms not completely known.

3.1. Control of the Apical Sodium Dependent Bile Acid Transporter (ASBT) by Polyphenols

ASBT is expressed in the ileum and the terminal portion of the small intestine, and is responsible for the re-circulation of 95% of BAs back to the liver [127]. Blocking BA enterohepatic circulation by interrupting the ASBT pathway increases hepatic BA synthesis to

compensate for BAs lost in the faeces [128]. Consequently, the concentration of liver cholesterol decreases causing the activation of compensatory mechanisms to maintain cellular cholesterol homeostasis such as increased hepatic uptake of LDL cholesterol, thereby, reducing circulating cholesterol levels [128]. Therefore, ASBT is an attractive therapeutic target to lower LDL cholesterol [129]. Inhibition of ASBT by SC-435, a potent ASBT inhibitor, was found to increase feacal BA excretion, upregulate *Cyp7a1* gene expression, decrease total cholesterol, and reduce the aortic lesion area in *Apo E-/-* mice [128]. Dietary cholesterol has direct effects on ASBT function and expression [130]. In vitro, 25-hydroxycholesterol, and to a lesser extent 22-and 24-hydroxycholesterol, reduced ASBT function and mRNA levels in Caco2 cells [131]. Moreover, 25-hydroxycholesterol significantly reduced the relative activity of the human ASBT promoter in a dose-dependent manner [131]. On the other hand, 100 μM cholesterol produced no inhibitory effect on sodium-dependent taurocholate uptake into COS-7 cells that were transiently transfected with ASBT [132]. In vivo, feeding 1% cholesterol to C57BL/6J female mice for two weeks decreased ASBT mRNA abundance by 54% when compared to control, while there was no change in ASBT protein expression in Sprague Dawley rats fed with 2% cholesterol for 10 days [132,133]. It is unlikely that the differences in these two studies were due to doses given, as in the later study, a much higher dose was given, which should be more effective than a 1% cholesterol dose. Possibly, the feeding duration could have caused these differences. However, in the later study using rats, changes in other genes such as *cyp7a1* were observed, indicating that there was ample feeding time to alter gene expression. As the time of cull was not specified in the later study, circadian gene expression changes may explain the observed differences [134]. Species differences could not be ruled out either, as in the same study that used rats, 2% cholesterol significantly increased ASBT protein expression in rabbits. Although cholesterol is not a dietary bioactive, the study results are applicable as polyphenols may alter cholesterol levels, which can further alter gene expression. However, for cholesterol, the results were varied, with a trend towards an enhanced cholesterol diet leading to a decrease in the expression of ASBT. Several reports have investigated the effects of dietary bioactives on the active transport of conjugated BAs via ASBT and these are summarised in Supplementary Table S3 The evidence that polyphenols such as resveratrol alter the gut microbiota to increase faecal BAs is further explored in the following sections of the review.

3.2. Changes in Gut Microbiota Can Increase Bile Acid Excretion: Action of Polyphenols

Microbial metabolism and de-conjugation of BAs lead to increased diversity within the BA pool and in general, a more hydrophobic population. Increasing the hydrophobic nature of the BAs makes them less readily re-absorbed and more easily excreted into faeces. De-conjugation of BAs is catalysed by bile salt hydrolase (BSH) enzymes and genes coding for *BSH* have been detected in the main bacterial genera: *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* [135]. In fact, BSH is enriched in the gut microbiota and helps the bacteria survive and colonise the gastrointestinal tract [136]. De-conjugated primary BAs that are not re-absorbed by the enterocytes enter the colon, where they are metabolised through 7-dehydroxylation into secondary BAs. 7-dehydroxylation of the primary BAs, CDCA, and CA can occur due to accessibility of the hydroxyl group, leading to DCA and LCA production. Bacteria that have 7-dehydroxylation activity are members of the *Firmicutes* phylum (*Clostridium* and *Eubacterium*) and these bacteria have a BA-inducible (BAI) gene. The bacteria are essential for the formation of secondary BAs and this is demonstrated in germ free mice, which have lower levels of secondary BAs and a smaller BA pool [137,138]. Germ-free animals accumulate cholesterol at higher levels [139], have higher levels of conjugated BAs, and significantly reduced excretion of faecal BAs [138,140].

The gut microbiota can also regulate BA metabolism by reducing the levels of tauro beta-muricholic acid (tbMCA), a naturally occurring FXR antagonist, and FXR antagonism increases *Cyp7a1* expression [137]. This has been further confirmed in a separate study, where an anti-oxidant, tempol, was used to suppress FXR signalling, which increased the levels of tbMCA [141]. Specifically, tempol decreased the genera *Lactobacillus* and *Clostridium*, which was accompanied by decreased BSH activity [141]. Oral feeding of *Lactobacillus plantarum* to mice resulted in significant reduction in LDL-C, increased faecal BA excretion, increased hepatic BA synthesis, and increased gene and protein expression of Cyp7a1 [142]. Recently, the glycine conjugate of bMCA (GbMCA) has also been found to be an FXR antagonist, which in contrast to TbMCA, was found to be resistant to faecal microbial BSH [143]. However, at present, it is not known whether TbMCA or GbMCA can antagonise FXR signalling in humans and how the human microbiota, which is more adapted to glycine conjugated BAs, metabolises GbMCA.

Human dietary interventions have shown that certain probiotics can reduce blood cholesterol by altering the microbial environment to increase BAs in the faeces [144,145]. The *Lactobacilus reuteri* strain, NCIMB 30242, which is known for its BSH activity reduced cholesterol levels in hypercholesterolaemic participants (>100) when fed an encapsulated form in a yoghurt. In a second study using the same strain of bacteria, but lyophylised, they found similar results and most importantly found that the participants had higher levels of de-conjugated BAs in their faeces [145]. In addition, live *Lactobacillus reuteri* with BSH activity when fed to pigs on a high fat, high cholesterol, low fibre diet reduced total and LDL-cholesterol concentrations [146]. Therefore, the interesting question is whether diet can alter the composition of the microbiota to decrease cholesterol levels.

Polyphenols have recently emerged as modulators of the gut microbiota [147]. Tea polyphenols have been shown to modulate the composition of the gut microbial community through the inhibition of pathogenic bacteria (*Clostridium perfringens*, *Clostridium difficile*, and *Bacteroides*) [148]. In the same study, no changes to the beneficial bacteria (*Clostridium*, *Bifidobacterium*, and *Lactobacillus)* were found [148], whereas, rats fed a diet containing tea polyphenols or gallotannins had reduced amounts of secondary BAs in the faeces, which suggests a reduction in bacteria with BSH activity [149]. Accordingly, in a study using rats, the supplementation of a high fat diet with catechin, curcumin, caffeic acid, rutin, ellagic acid, or quercetin reduced the concentration of secondary BAs in the faeces [150]. This suggests that the amount of de-conjugating bacteria were also reduced in the rats on these diets [150]. However, when tea flavan-3-ol monomers such as catechin were fed to humans, there was an enhanced growth of members of *Clostridium coccoides*, particularly *Eubacterium rectale*, which are known to break down BAs to secondary BAs [151]. Interestingly, *Eubacterium rectale* spp. has been shown to play a probiotic role of butyrate synthesis from carbohydrates [152–154] and other members of the *Clostridium coccoides* family are known to have high levels of BA 7α-dehydroxylating activity, which break down primary BAs to secondary BAs [153].

A human intervention study has shown that consumption of red wine polyphenols significantly increased the number of *Bacteroides*, *Bifidobacterium*, *Enterococcus*, *Prevotella*, *Bacteroides uniformis*, *Eggerthellalenta*, and *Blautia coccoides-E. rectale*. Correspondingly, total cholesterol was significantly decreased and most importantly, changes in cholesterol concentrations were linked to changes in the *Bifidobacteria* number, *Bifidobacteria* were probiotic, and some groups had BSH activity, although BSH activity was not measured [152]. This corresponded to a study in rats supplemented with a de-alcoholised, proanthocyanidin-rich red wine extract over 16-weeks, where the bacterial composition changed from a predominance of *Bacteroides*, *Clostridium*, and *Propionibacterium* to a prevalence of *Bacteroides*, *Lactobacillus,* and *Bifidobacterium* [155]. Interestingly, in another study, red wine tannins had no effect on the conversion of primary to secondary BAs in the faeces of rats [156].

In a human intervention study, the consumption of a wild blueberry drink, rich in polyphenols significantly increased the amount of *Bifidobacterium*, some *Bifidobacteria* are known to have BSH activity [157]. Proanthocyanidin-rich extract from grape seeds fed to healthy adults for two weeks also significantly increased the number of intestinal *Bifidobacteria* [158]. In addition, resveratrol, which is found in wine and grapes, also increased faecal counts of *Bifidobacterium* and increased *Lactobacillus* in a rat model, and in humans, it increased α-diversity such as *Barnesiella* levels that are associated with gut health and may improve cholesterol metabolism [159,160]. In one study, which substituted the water fed to rats for grape, apple, or beetroot juice, a higher concentration of primary BAs but lower concentration of secondary BAs was found in the intestinal contents along with increased amounts of cholesterol and its metabolites. Intriguingly, this corresponded to increased faecal counts of bacteria with BSH activity, *Lactobacillus* and *Bifidobacterium* [114]. Raspberry pomace containing seeds fed to rats on a high fat diet also reduced the amount of secondary BAs (LCA and DCA) in the cecum, but interestingly, the seedless fraction did not have the same effect [161]. Grape seed flour has been shown to alter the gut microbiota of male Golden Syrian hamsters. Hamsters were fed a high-fat (HF) control diet or a HF diet supplemented with 10% partially defatted grape seed flour. The Chardonnay diet altered the numbers of total bacteria and relative abundances of *Bifidobacterium*, *Lactobacillus*, and *Firmicutes* in the faeces, which were significantly lower than the control group. This was accompanied by decreased intestinal FGF15 expression and increased liver *Cyp7a1* gene expression [162]. It was suggested that alteration of the intestinal microbiota may regulate BA metabolism, but BAs were not measured in this study.

4. Conclusions

4.1. The Limitations of Existing Models

Rodent models are useful for studying the effects of polyphenols on faecal BAs, liver, and circulating cholesterol levels, but the evidence that this occurs through the regulation of CYP7A1 in humans should be examined with caution, as human cells lack a LXRα response element in the human CYP7A1 promoter. Nevertheless, the effects of polyphenols on FXR signalling are similar in mice and humans with studies showing that polyphenols affect this signalling pathway in both species [96,97]. The reverse cholesterol transport pathway and the NF-κB and ERK signalling pathway are also both inducible by polyphenols in humans and mice [71,77,163,164], providing a degree of confidence that effects observed in mouse models would also be observed in humans. Thus, indicating great potential for observations in rodent models to inform future translational studies in human. Human cell lines are the obvious next best choice for functional studies, but previous research has shown that cell density can affect the expression of *CYP7A1*mRNA [165], and it has been shown that *CYP7A1* expression is dependent on hepatocyte differentiation [166]. Specifically, cells which are more terminally differentiated are more responsive to hormones or BA conjugates when compared to less differentiated cells [13]. *CYP7A1* mRNA levels significantly increased in HepG2 cells cultured over time [14]. In addition, the presence of Fetal Bovine Serum (FBS) inhibits *CYP7A1*expression due to the presence of bovine BAs [14]. The removal of serum was shown to stimulate *CYP7A1* mRNA levels in HepG2 cells [13]. A factor in the serum such as calf BAs may be directly repressing expression of *Cyp7a1.* Alternatively, serum may influence hepatocyte differentiation, as serum-free medium has been found to promote a more differentiated phenotype than serum-containing medium [167,168]. Additionally, the presence of LDL cholesterol can affect the impact on *CYP7A1.* For example, when HepG2 cells were incubated in serum free medium with or without a red grape juice (RGJ) extract, different results were observed. In cells exposed to LDL-C, RGJ caused a marked reduction in the

expression of *CYP7A1* expression, however, when LDL-C was absent, increased *CYP7A1* levels were observed on treatment with RGJ [169]. Therefore, care in the interpretation of both human and animal studies is required. Additionally, cells lines do not represent a multi-organ regulation of cholesterol and bile acid metabolism as do animal studies. Another factor that may greatly lead to disparities between experiments may be the concentrations of polyphenols used, some of which may not be physiologically relevant.

4.2. The Most Promising Polyphenol Candidates for Future Studies

Puerarin, an isoflavonoid, is a promising candidate for further studies. Rodent studies from three separate labs have shown that puerarin increases hepatic *Cyp7a1* expression [81–83]. However, when liver HepG2 cells were treated with increasing doses of puerarin, there was no effect, highlighting the disparity between human and animal studies [83]. The predominant mechanism for BA induction via puerarin in mice appears to be LXRα driven, which suggests that BA induction may not occur in human cells via the same pathway. This suggests that BA induction via polyphenols is not always LXRα driven. Quercetin has also been shown to regulate cholesterol metabolism via LXRα signalling as well as reverse cholesterol transport, which also occurs in humans, making it a promising candidate for further studies in humans [163]. There are documented mechanisms whereby polyphenols can induce CYP7A1 expression that are not driven by LXRα. For example, NF-κB and ERK signalling induces *CYP7A1*expression in human cells treated with naringin, a flavanone-7-O-glycoside [77]. In addition, attenuated FXR signalling by resveratrol, a stilbene, has been shown to induce *CYP7A1* mRNA expression in human HepG2 liver cells [91,92,97]. In conclusion, there is some promising research that polyphenols may regulate CYP7A1 expression. In addition, modification of the gut bacteria appears to be an important factor in the enhancement of BAs in the faeces, through the enrichment of bacteria with BSH activity. Red wine, rich in phenolic polymers and resveratrol, has been particularly well documented at increasing beneficial bacteria [152,155,159]. In particular, resveratrol increased levels of *Lactobacillus* and *Bifidobacterium* in mice and bacterial α-diversity in humans. These bacteria have enhanced BSH activity, which enables increased BA de-conjugation to promote faecal excretion of BAs [98]. Further studies are required to ascertain whether the parent polyphenols or their metabolites are responsible for the alterations in the gut microbiota and whether the effects seen in animal studies can be replicated in humans to inform future clinical studies.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: The categories and structures of dietary polyphenols. Polyphenols are subdivided into different categories: flavonoids, isoflavonoids, lignans, stilbenes, phenolic acids, and phenolic polymers. Table S1: A list of all the known BAs from rodents and humans with their abbreviations used in the review. * Dehydrocholic acid is a synthetic BA, manufactured by the oxidation of cholic acid. Table S2: The published studies that have determined whether dietary polyphenols can alter BA excretion and *Cyp7a1* expression. NR = not reported. Table S3: Effects of polyphenols on ASBT protein and gene expression. NR = not reported.

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Abbreviations:

BA	bile acid
Cyp7a1	cholesterol 7 alpha-hydroxylase
RCT	reverse cholesterol transport
HDL	high-density lipoprotein
LDL	low-density lipoprotein
FXR	farnesoid X receptor
BSH	bile salt hydrolase
HF	high-fat
CDCA	chenodeoxycholic acid

References

1. Gistera, A.; Hansson, G.K. The immunology of atherosclerosis. *Nat. Rev. Nephrol.* **2017**, *13*, 368–380, doi:10.1038/nrneph.2017.51.
2. Russell, D.W. The enzymes, regulation, and genetics of bile acid synthesis. *Annu. Rev. Biochem.* **2003**, *72*, 137–174, doi:10.1146/annurev.biochem.72.121801.161712.
3. Schwarz, M.; Russell, D.W.; Dietschy, J.M.; Turley, S.D. Marked reduction in bile acid synthesis in cholesterol 7alpha-hydroxylase-deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolemia. *J. Lipid Res.* **1998**, *39*, 1833–1843.
4. Duane, W.C.; Javitt, N.B. 27-hydroxycholesterol: Production rates in normal human subjects. *J. Lipid Res.* **1999**, *40*, 1194–1199.
5. De Aguiar Vallim, T.Q.; Tarling, E.J.; Edwards, P.A. Pleiotropic Roles of Bile Acids in Metabolism. *Cell Metab.* **2013**, *17*, 657–669, doi:10.1016/j.cmet.2013.03.013.
6. Falany, C.N.; Johnson, M.R.; Barnes, S.; Diasio, R.B. Glycine and taurine conjugation of bile acids by a single enzyme. Molecular cloning and expression of human liver bile acid CoA: Amino acid N-acyltransferase. *J. Biol. Chem.* **1994**, *269*, 19375–19379.
7. Hayashi, H.; Takada, T.; Suzuki, H.; Onuki, R.; Hofmann, A.F.; Sugiyama, Y. Transport by vesicles of glycine- and taurine-conjugated bile salts and taurolithocholate 3-sulfate: A comparison of human BSEP with rat Bsep. *Biochim. Biophys. Acta* **2005**, *1738*, 54–62, doi:10.1016/j.bbalip.2005.10.006.
8. Dawson, P.A.; Lan, T.; Rao, A. Bile acid transporters. *J. Lipid Res.* **2009**, *50*, 2340–2357, doi:10.1194/jlr.R900012-JLR200.
9. Charlton-Menys, V.; Durrington, P.N. Human cholesterol metabolism and therapeutic molecules. *Exp. Physiol.* **2008**, *93*, 27–42, doi:10.1113/expphysiol.2007.035147.
10. Peet, D.J.; Turley, S.D.; Ma, W.; Janowski, B.A.; Lobaccaro, J.M.; Hammer, R.E.; Mangelsdorf, D.J. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* **1998**, *93*, 693–704.
11. Jelinek, D.F.; Andersson, S.; Slaughter, C.A.; Russell, D.W. Cloning and regulation of cholesterol 7 alpha-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. *J. Biol. Chem.* **1990**, *265*, 8190–8197.
12. Noshiro, M.; Nishimoto, M.; Okuda, K. Rat liver cholesterol 7 alpha-hydroxylase. Pretranslational regulation for circadian rhythm. *J. Biol. Chem.* **1990**, *265*, 10036–10041.
13. Taniguchi, T.; Chen, J.; Cooper, A.D. Regulation of cholesterol 7 alpha-hydroxylase gene expression in Hep-G2 cells. Effect of serum, bile salts, and coordinate and noncoordinate regulation with other sterol-responsive genes. *J. Biol. Chem.* **1994**, *269*, 10071–10078.
14. Andreou, E.R. *Analysis of CYP7A1 Gene Regulation in HepG2 Cells by Reverse-Transcriptase Polymerase Chain Reaction*; University of Toronto: Toronto, Canada, 1997.

15. Hirsch, N.; Konstantinov, A.; Anavi, S.; Aronis, A.; Hagay, Z.; Madar, Z.; Tirosh, O. Prolonged feeding with green tea polyphenols exacerbates cholesterol-induced fatty liver disease in mice. *Mol. Nutr. Food Res.* **2016**, doi:10.1002/mnfr.201600221.
16. Tiemann, M.; Han, Z.; Soccio, R.; Bollineni, J.; Shefer, S.; Sehayek, E.; Breslow, J.L. Cholesterol feeding of mice expressing cholesterol 7alpha-hydroxylase increases bile acid pool size despite decreased enzyme activity. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 1846–1851, doi:10.1073/pnas.0308426100.
17. Eggink, H.M.; Oosterman, J.E.; De Goede, P.; De Vries, E.M.; Foppen, E.; Koehorst, M.; Groen, A.K.; Boelen, A.; Romijn, J.A.; La Fleur, S.E.; et al. Complex interaction between circadian rhythm and diet on bile acid homeostasis in male rats. *Chronobiol. Int.* **2017**, *34*, 1339–1353, doi:10.1080/07420528.2017.1363226.
18. Chen, P.P.; Zhang, R.Y.; Mou, L.J.; Li, X.W.; Qin, Y.; Li, X.M. An impaired hepatic clock system effects lipid metabolism in rats with nephropathy. *Int. J. Mol. Med.* **2018**, *42*, 2720–2736, doi:10.3892/ijmm.2018.3833.
19. Story, J.A.; Watterson, J.J.; Matheson, H.B.; Furumoto, E.J. Dietary Fiber and Bile-Acid Metabolism. *Adv. Exp. Med. Biol.* **1990**, *270*, 43–48.
20. Ide, T. Dietary Fiber-Induced Changes in Bile-Acid Conjugation and Taurine Metabolism in Rats. In *Food Hydrocolloids*; Springer: Boston, MA, USA, 1993; pp. 491–496.
21. Locket, P.; Gallaher, D. The Effect of Fiber Source and Fat Level on Bile-Acid Metabolism in the Rat. *Faseb J.* **1988**, *2*, A862–A862.
22. Fadden, K.; Hill, M.J.; Owen, R.W. Effect of fibre on bile acid metabolism by human faecal bacteria in batch and continuous culture. *Eur. J. Cancer Prev.* **1997**, *6*, 175–194.
23. Kritchevsky, D. Influence of Dietary Fiber on Bile-Acid Metabolism. *Lipids* **1978**, *13*, 982–985, doi:10.1007/Bf02533860.
24. Calkin, A.C.; Tontonoz, P. Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 213–224, doi:10.1038/nrm3312.
25. Lee, E.A.; Lee, D.I.; Kim, H.Y.; Ahn, S.H.; Seong, H.R.; Jung, W.H.; Kim, K.Y.; Kim, S.; Rhee, S.D. Cyp7a1 is continuously increased with disrupted Fxr-mediated feedback inhibition in hypercholesterolemic TALLYHO/Jng mice. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2018**, *1863*, 20–25, doi:10.1016/j.bbalip.2017.08.007.
26. Chiang, J.Y.; Kimmel, R.; Weinberger, C. Stroup D: Farnesoid X receptor responds to bile acids and represses cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription. *J. Biol. Chem.* **2000**, *275*, 10918–10924.
27. Li, T.; Chiang, J.Y. Mechanism of rifampicin and pregnane X receptor inhibition of human cholesterol 7 alpha-hydroxylase gene transcription. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2005**, *288*, G74–G84, doi:10.1152/ajpgi.00258.2004.
28. Goodwin, B.; Watson, M.A.; Kim, H.; Miao, J.; Kemper, J.K.; Kliewer, S.A. Differential regulation of rat and human CYP7A1 by the nuclear oxysterol receptor liver X receptor-alpha. *Mol. Endocrinol.* **2003**, *17*, 386–394, doi:10.1210/me.2002-0246.
29. Shin, D.J.; Campos, J.A.; Gil, G.; Osborne, T.F. PGC-1alpha activates CYP7A1 and bile acid biosynthesis. *J. Biol. Chem.* **2003**, *278*, 50047–50052, doi:10.1074/jbc.M309736200.
30. Kir, S.; Zhang, Y.; Gerard, R.D.; Kliewer, S.A.; Mangelsdorf, D.J. Nuclear receptors HNF4alpha and LRH-1 cooperate in regulating Cyp7a1 in vivo. *J. Biol. Chem.* **2012**, *287*, 41334–41341, doi:10.1074/jbc.M112.421834.
31. Marrapodi, M.; Chiang, J.Y. Peroxisome proliferator-activated receptor alpha (PPARalpha) and agonist inhibit cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription. *J. Lipid Res.* **2000**, *41*, 514–520.
32. Inoue, Y.; Yu, A.M.; Yim, S.H.; Ma, X.; Krausz, K.W.; Inoue, J.; Xiang, C.C.; Brownstein, M.J.; Eggertsen, G.; Bjorkhem, I.; Gonzalez, F.J. Regulation of bile acid biosynthesis by hepatocyte nuclear factor 4alpha. *J. Lipid Res.* **2006**, *47*, 215–227.

33. Patel, D.D.; Knight, B.L.; Soutar, A.K.; Gibbons, G.F.; Wade, D.P. The effect of peroxisome-proliferator-activated receptor-alpha on the activity of the cholesterol 7 alpha-hydroxylase gene. *Biochem. J.* **2000**, *351 Pt 3*, 747–753.
34. Duan, Y.; Chen, Y.; Hu, W.; Li, X.; Yang, X.; Zhou, X.; Yin, Z.; Kong, D.; Yao, Z.; Hajjar, D.P.; et al. Peroxisome Proliferator-activated receptor gamma activation by ligands and dephosphorylation induces proprotein convertase subtilisin kexin type 9 and low density lipoprotein receptor expression. *J. Biol. Chem.* **2012**, *287*, 23667–23677, doi:10.1074/jbc.M112.350181.
35. Li, T.; Kong, X.; Owsley, E.; Ellis, E.; Strom, S.; Chiang, J.Y. Insulin regulation of cholesterol 7alpha-hydroxylase expression in human hepatocytes: Roles of forkhead box O1 and sterol regulatory element-binding protein 1c. *J. Biol. Chem.* **2006**, *281*, 28745–28754, doi:10.1074/jbc.M605815200.
36. Park, W.H.; Pak, Y.K. Insulin-dependent suppression of cholesterol 7alpha-hydroxylase is a possible link between glucose and cholesterol metabolisms. *Exp. Mol. Med.* **2011**, *43*, 571–579, doi:10.3858/emm.2011.43.10.064.
37. Le Martelot, G.; Claudel, T.; Gatfield, D.; Schaad, O.; Kornmann, B.; Lo Sasso, G.; Moschetta, A.; Schibler, U. REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biol.* **2009**, *7*, e1000181, doi:10.1371/journal.pbio.1000181.
38. Zhang, T.; Zhao, M.; Lu, D.; Wang, S.; Yu, F.; Guo, L.; Wen, S.; Wu, B. REV-ERBalpha Regulates CYP7A1 Through Repression of Liver Receptor Homolog-1. *Drug Metab. Dispos.* **2018**, *46*, 248–258, doi:10.1124/dmd.117.078105.
39. Lee, Y.H.; Alberta, J.A.; Gonzalez, F.J.; Waxman, D.J. Multiple, functional DBP sites on the promoter of the cholesterol 7 alpha-hydroxylase P450 gene, CYP7. Proposed role in diurnal regulation of liver gene expression. *J. Biol. Chem.* **1994**, *269*, 14681–14689.
40. Ogawa, A.; Yano, M.; Tsujinaka, T.; Morimoto, T.; Morita, S.; Taniguchi, M.; Shiozaki, H.; Okamoto, K.; Sato, S.; Monden, M. Modulation of circadian expression of D-site binding protein by the schedule of parenteral nutrition in rat liver. *Hepatology* **1997**, *26*, 1580–1586, doi:10.1053/jhep.1997.v26.pm0009398001.
41. Le Martelot, G.; Claudel, T.; Gatfield, D.; Schaad, O.; Kornmann, B.; Sasso, G.L.; Moschetta, A.; Schibler, U. REV-ERBα Participates in Circadian SREBP Signaling and Bile Acid Homeostasis. *PLoS Biol.* **2009**, *7*, e1000181, doi:10.1371/journal.pbio.1000181.
42. Berard, A.M.; Dumon, M.F.; Darmon, M. Dietary fish oil up-regulates cholesterol 7alpha-hydroxylase mRNA in mouse liver leading to an increase in bile acid and cholesterol excretion. *FEBS Lett.* **2004**, *559*, 125–128, doi:10.1016/s0014-5793(04)00049-3.
43. Chiang, J.Y.L. Bile acids: Regulation of synthesis. *J. Lipid Res.* **2009**, *50*, 1955–1966, doi:10.1194/jlr.R900010-JLR200.
44. Li, T.; Francl, J.M.; Boehme, S.; Chiang, J.Y. Regulation of cholesterol and bile acid homeostasis by the cholesterol 7alpha-hydroxylase/steroid response element-binding protein 2/microRNA-33a axis in mice. *Hepatology* **2013**, *58*, 1111–1121, doi:10.1002/hep.26427.
45. Song, K.H.; Li, T.; Owsley, E.; Chiang, J.Y. A putative role of micro RNA in regulation of cholesterol 7alpha-hydroxylase expression in human hepatocytes. *J. Lipid Res.* **2010**, *51*, 2223–2233, doi:10.1194/jlr.M004531.
46. Rodrigues, P.M.; Afonso, M.B.; Simao, A.L.; Carvalho, C.C.; Trindade, A.; Duarte, A.; Borralho, P.M.; Machado, M.V.; Cortez-Pinto, H.; Rodrigues, C.M.; et al. miR-21 ablation and obeticholic acid ameliorate nonalcoholic steatohepatitis in mice. *Cell Death Dis.* **2017**, *8*, e2748, doi:10.1038/cddis.2017.172.
47. Gong, R.; Lv, X.; Liu, F. MiRNA-17 encoded by the miR-17-92 cluster increases the potential for steatosis in hepatoma cells by targeting CYP7A1. *Cell Mol. Biol. Lett.* **2018**, *23*, 16, doi:10.1186/s11658-018-0083-3.
48. Takagi, S.; Nakajima, M.; Kida, K.; Yamaura, Y.; Fukami, T.; Yokoi, T. MicroRNAs regulate human hepatocyte nuclear factor 4alpha, modulating the expression of metabolic enzymes and cell cycle. *J. Biol. Chem.* **2010**, *285*, 4415–4422, doi:10.1074/jbc.M109.085431.

49. Wang, D.; Gao, Q.; Wang, T.; Zhao, G.; Qian, F.; Huang, J.; Wang, H.; Zhang, X.; Wang, Y. Green tea infusion protects against alcoholic liver injury by attenuating inflammation and regulating the PI3K/Akt/eNOS pathway in C57BL/6 mice. *Food Funct.* **2017**, *8*, 3165–3177, doi:10.1039/c7fo00791d.
50. Kawabata, K.; Yoshioka, Y.; Terao, J. Role of Intestinal Microbiota in the Bioavailability and Physiological Functions of Dietary Polyphenols. *Molecules* **2019**, *24*, doi:10.3390/molecules24020370.
51. Teng, H.; Chen, L. Polyphenols and bioavailability: An update. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 2040–2051, doi:10.1080/10408398.2018.1437023.
52. D'Archivio, M.; Filesi, C.; Vari, R.; Scazzocchio, B.; Masella, R. Bioavailability of the polyphenols: Status and controversies. *Int. J. Mol. Sci.* **2010**, *11*, 1321–1342, doi:10.3390/ijms11041321.
53. Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S, doi:10.1093/ajcn/81.1.230S.
54. Yahfoufi, N.; Alsadi, N.; Jambi, M.; Matar, C. The Immunomodulatory and Anti-Inflammatory Role of Polyphenols. *Nutrients* **2018**, *10*, doi:10.3390/nu10111618.
55. Chu, A.J. Antagonism by bioactive polyphenols against inflammation: A systematic view. *Inflamm. Allergy Drug Targets* **2014**, *13*, 34–64.
56. EFSA. Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage. *EFSA J.* **2011**, doi:10.2903/j.efsa.2011.2033.
57. Millar, C.L.; Duclos, Q.; Blesso, C.N. Effects of Dietary Flavonoids on Reverse Cholesterol Transport, HDL Metabolism, and HDL Function. *Adv. Nutr.* **2017**, *8*, 226–239, doi:10.3945/an.116.014050.
58. Yang, T.T.; Koo, M.W. Chinese green tea lowers cholesterol level through an increase in fecal lipid excretion. *Life Sci.* **2000**, *66*, 411–423.
59. Hirsch, N.; Konstantinov, A.; Anavi, S.; Aronis, A.; Hagay, Z.; Madar, Z.; Tirosh, O. Prolonged feeding with green tea polyphenols exacerbates cholesterol-induced fatty liver disease in mice. *Mol. Nutr. Food Res.* **2016**, *60*, 2542–2553, doi:10.1002/mnfr.201600221.
60. Hirsova, P.; Karlasova, G.; Dolezelova, E.; Cermanova, J.; Zagorova, M.; Kadova, Z.; Hroch, M.; Sispera, L.; Tomsik, P.; Lenicek, M.; et al. Cholestatic effect of epigallocatechin gallate in rats is mediated via decreased expression of Mrp2. *Toxicology* **2013**, *303*, 9–15, doi:10.1016/j.tox.2012.10.018.
61. Shan, D.; Fang, Y.; Ye, Y.; Liu, J. EGCG reducing the susceptibility to cholesterol gallstone formation through the regulation of inflammation. *Biomed. Pharmacother.* **2008**, *62*, 677–683, doi:10.1016/j.biopha.2007.12.008.
62. Lee, M.S.; Park, J.Y.; Freake, H.; Kwun, I.S.; Kim, Y. Green tea catechin enhances cholesterol 7alpha-hydroxylase gene expression in HepG2 cells. *Br. J. Nutr.* **2008**, *99*, 1182–1185.
63. Li, G.; Lin, W.; Araya, J.J.; Chen, T.; Timmermann, B.N.; Guo, G.L. A tea catechin, epigallocatechin-3-gallate, is a unique modulator of the farnesoid X receptor. *Toxicol. Appl. Pharmacol.* **2012**, *258*, 268–274, doi:10.1016/j.taap.2011.11.006.
64. Park, Y.; Park, E.M.; Kim, E.H.; Chung, I.M. Hypocholesterolemic metabolism of dietary red pericarp glutinous rice rich in phenolic compounds in mice fed a high cholesterol diet. *Nutr. Res. Pract.* **2014**, *8*, 632–637, doi:10.4162/nrp.2014.8.6.632.
65. Matziouridou, C.; Marungruang, N.; Nguyen, T.D.; Nyman, M.; Fak, F. Lingonberries reduce atherosclerosis in Apoe(-/-) mice in association with altered gut microbiota composition and improved lipid profile. *Mol. Nutr. Food Res.* **2016**, *60*, 1150–1160, doi:10.1002/mnfr.201500738.
66. Kim, S.J.; Maeda, T.; Sarker, M.Z.; Takigawa, S.; Matsuura-Endo, C.; Yamauchi, H.; Mukasa, Y.; Saito, K.; Hashimoto, N.; Noda, T.; et al. Identification of anthocyanins in the sprouts of buckwheat. *J. Agric. Food Chem.* **2007**, *55*, 6314–6318, doi:10.1021/jf0704716.
67. Watanabe, M.; Ayugase, J. Effects of buckwheat sprouts on plasma and hepatic parameters in type 2 diabetic db/db mice. *J. Food Sci.* **2010**, *75*, H294–H299, doi:10.1111/j.1750-3841.2010.01853.x.
68. Wang, D.; Xia, M.; Gao, S.; Li, D.; Zhang, Y.; Jin, T.; Ling, W. Cyanidin-3-O-beta-glucoside upregulates hepatic cholesterol 7alpha-hydroxylase expression and reduces hypercholesterolemia in mice. *Mol. Nutr. Food Res.* **2012**, *56*, 610–621, doi:10.1002/mnfr.201100659.

69. Zhang, C.; Zhang, R.; Li, Y.M.; Liang, N.; Zhao, Y.; Zhu, H.; He, Z.; Liu, J.; Hao, W.; Jiao, R., et al. Cholesterol-Lowering Activity of Tartary Buckwheat Protein. *J. Agric. Food Chem.* **2017**, *65*, 1900–1906, doi:10.1021/acs.jafc.7b00066.
70. Huang, J.B.; Feng, S.M.; Liu, A.N.; Dai, Z.Q.; Wang, H.; Reuhl, K.; Lu, W.Y.; Yang, C.S. Green Tea Polyphenol EGCG Alleviates Metabolic Abnormality and Fatty Liver by Decreasing Bile Acid and Lipid Absorption in Mice. *Mol. Nutr. Food Res.* **2018**, *62*, doi:10.1002/mnfr.201700696.
71. Zhang, M.; Xie, Z.; Gao, W.; Pu, L.; Wei, J.; Guo, C. Quercetin regulates hepatic cholesterol metabolism by promoting cholesterol-to-bile acid conversion and cholesterol efflux in rats. *Nutr. Res.* **2016**, *36*, 271–279, doi:10.1016/j.nutres.2015.11.019.
72. Chavez-Santoscoy, R.A.; Gutierrez-Uribe, J.A.; Granados, O.; Torre-Villalvazo, I.; Serna-Saldivar, S.O.; Torres, N.; Palacios-González, B.; Tovar, A.R. Flavonoids and saponins extracted from black bean (*Phaseolus vulgaris* L.) seed coats modulate lipid metabolism and biliary cholesterol secretion in C57BL/6 mice. *Br. J. Nutr.* **2014**, *112*, 886–899, doi:10.1017/S0007114514001536.
73. Daniel, R.S.; Devi, K.S.; Augusti, K.T.; Sudhakaran Nair, C.R. Mechanism of action of antiatherogenic and related effects of Ficus bengalensis Linn. flavonoids in experimental animals. *Indian J. Exp. Biol.* **2003**, *41*, 296–303.
74. Mathew, B.C.; Yoseph, B.A.; Dessale, T.; Daniel, R.S.; Alemayehu, A.; Campbell, I.W.; Augusti, K.T. Hypolipidaemic effect of Leucodelphinidin derivative from Ficus bengalensis Linn on Cholesterol fed rats. *Res. J. Chem. Sci.* **2012**, *2*, 54–60.
75. Hoang, M.H.; Jia, Y.; Mok, B.; Jun, H.J.; Hwang, K.Y.; Lee, S.J. Kaempferol ameliorates symptoms of metabolic syndrome by regulating activities of liver X receptor-beta. *J. Nutr. Biochem.* **2015**, *26*, 868–875, doi:10.1016/j.jnutbio.2015.03.005.
76. Goldwasser, J.; Cohen, P.Y.; Yang, E.; Balaguer, P.; Yarmush, M.L.; Nahmias, Y. Transcriptional Regulation of Human and Rat Hepatic Lipid Metabolism by the Grapefruit Flavonoid Naringenin: Role of PPARα, PPARγ and LXRα. *PLoS ONE* **2010**, *5*, e12399, doi:10.1371/journal.pone.0012399.
77. Liang, J.; Wang, C.; Peng, J.; Li, W.; Jin, Y.; Liu, Q.; Meng, Q.; Liu, K.; Sun, H. Naringin regulates cholesterol homeostasis and inhibits inflammation via modulating NF-kappaB and ERK signaling pathways in vitro. *Die Pharm.* **2016**, *71*, 101–108.
78. Lin, H.R.; Chou, T.H.; Huang, D.W.; Chen, I.S. Cryptochinones from Cryptocarya chinensis act as farnesoid X receptor agonists. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 4181–4186, doi:10.1016/j.bmcl.2014.07.045.
79. de Almeida Jackix, E.; Monteiro, E.B.; Raposo, H.F.; Vanzela, E.C.; Amaya-Farfan, J. *Taioba* (*Xanthosoma sagittifolium*) leaves: Nutrient composition and physiological effects on healthy rats. *J. Food Sci.* **2013**, *78*, H1929-1934, doi:10.1111/1750-3841.12301.
80. Picerno, P.; Mencherini, T.; Lauro, M.R.; Barbato, F.; Aquino, R. Phenolic Constituents and Antioxidant Properties of Xanthosoma violaceum Leaves. *J. Agric. Food Chem.* **2003**, *51*, 6423–6428, doi:10.1021/jf030284h.
81. Ma, J.Q.; Ding, J.; Zhao, H.; Liu, C.M. Puerarin attenuates carbon tetrachloride-induced liver oxidative stress and hyperlipidaemia in mouse by JNK/c-Jun/CYP7A1 pathway. *Basic Clin. Pharmacol. Toxicol.* **2014**, *115*, 389–395, doi:10.1111/bcpt.12245.
82. Yan, L.P.; Chan, S.W.; Chan, A.S.; Chen, S.L.; Ma, X.J.; Xu, H.X. Puerarin decreases serum total cholesterol and enhances thoracic aorta endothelial nitric oxide synthase expression in diet-induced hypercholesterolemic rats. *Life Sci.* **2006**, *79*, 324–330, doi:10.1016/j.lfs.2006.01.016.
83. Chung, M.J.; Sung, N.-J.; Park, C.-S.; Kweon, D.-K.; Mantovani, A.; Moon, T.-W.; Lee, S.-J.; Park, K.-H. Antioxidative and hypocholesterolemic activities of water-soluble puerarin glycosides in HepG2 cells and in C57 BL/6J mice. *Eur. J. Pharmacol.* **2008**, *578*, 159–170, doi:10.1016/j.ejphar.2007.09.036.
84. Feng, D.; Sun, J.G.; Sun, R.B.; Ou-Yang, B.C.; Yao, L.; Aa, J.Y.; Zhou, F.; Zhang, J.W.; Zhang, J.; Wang, G.J. Isoflavones and phytosterols contained in Xuezhikang capsules modulate cholesterol homeostasis in high-fat diet mice. *Acta Pharmacol. Sin.* **2015**, *36*, 1462–1472, doi:10.1038/aps.2015.98.
85. Mvondo, M.A.; Njamen, D.; Kretzschmar, G.; Imma Bader, M.; Tanee Fomum, S.; Wandji, J.; Vollmer, G. Alpinumisoflavone and abyssinone V 4′-methylether derived from *Erythrina lysistemon*

(Fabaceae) promote HDL-cholesterol synthesis and prevent cholesterol gallstone formation in ovariectomized rats. *J. Pharm. pharmacol.* **2015**, *67*, 990–996, doi:10.1111/jphp.12386.

86. Ni, Y.D.; Wei, X.J.; Zhang, C.X.; Zhong, Y.; Lu, L.Z.; Grossmann, R.; Zhao, R.Q. The effect of equol injection in ovo on lipid metabolism and hepatic lipogenic gene expression in broilers. *Anim. Int. J. Anim. Biosci.* **2012**, *6*, 1444–1450, doi:10.1017/s1751731112000468.
87. Kobayashi, M.; Harada, T.; Takagi, N.; Tsuzuki, K.; Sugawara, M.; Fukuda, M. Effects of lactic acid-fermented soymilk on lipid metabolism-related gene expression in rat liver. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 19–24, doi:10.1271/bbb.100354.
88. Zhang, H.; Bartley, G.E.; Mitchell, C.R.; Zhang, H.; Yokoyama, W. Lower weight gain and hepatic lipid content in hamsters fed high fat diets supplemented with white rice protein, brown rice protein, soy protein, and their hydrolysates. *J. Agric. Food Chem.* **2011**, *59*, 10927–10933, doi:10.1021/jf202721z.
89. Laurent, T.; Okuda, Y.; Chijimatsu, T.; Umeki, M.; Kobayashi, S.; Kataoka, Y.; Tatsuguchi, I.; Mochizuki, S.; Oda, H. Freshwater Clam Extract Ameliorates Triglyceride and Cholesterol Metabolism through the Expression of Genes Involved in Hepatic Lipogenesis and Cholesterol Degradation in Rats. *Evid.-Based Complement. Altern. Med. eCAM* **2013**, *2013*, 830684, doi:10.1155/2013/830684.
90. Kroon, P.A.; Iyer, A.; Chunduri, P.; Chan, V.; Brown, L. The cardiovascular nutrapharmacology of resveratrol: Pharmacokinetics, molecular mechanisms and therapeutic potential. *Curr. Med. Chem.* **2010**, *17*, 2442–2455.
91. Chen, Q.; Wang, E.; Ma, L.; Zhai, P. Dietary resveratrol increases the expression of hepatic 7alpha-hydroxylase and ameliorates hypercholesterolemia in high-fat fed C57BL/6J mice. *Lipids Health Dis.* **2012**, *11*, 56, doi:10.1186/1476-511x-11-56.
92. Shao, D.; Wang, Y.; Huang, Q.; Shi, J.; Yang, H.; Pan, Z.; Jin, M.; Zhao, H.; Xu, X. Cholesterol-Lowering Effects and Mechanisms in View of Bile Acid Pathway of Resveratrol and Resveratrol Glucuronides. *J. Food Sci.* **2016**, doi:10.1111/1750-3841.13528.
93. Wang, T.; Zhou, Z.X.; Sun, L.X.; Li, X.; Xu, Z.M.; Chen, M.; Zhao, G.L.; Jiang, Z.Z.; Zhang, L.Y. Resveratrol effectively attenuates alpha-naphthyl-isothiocyanate-induced acute cholestasis and liver injury through choleretic and anti-inflammatory mechanisms. *Acta Pharmacol. Sin.* **2014**, *35*, 1527–1536, doi:10.1038/aps.2014.119.
94. Miura, D.; Miura, Y.; Yagasaki, K. Hypolipidemic action of dietary resveratrol, a phytoalexin in grapes and red wine, in hepatoma-bearing rats. *Life Sci.* **2003**, *73*, 1393–1400.
95. Marier, J.F.; Vachon, P.; Gritsas, A.; Zhang, J.; Moreau, J.P.; Ducharme, M.P. Metabolism and disposition of resveratrol in rats: Extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. *J. Pharmacol. Exp. Ther.* **2002**, *302*, 369–373.
96. Qiao, Y.; Sun, J.; Xia, S.; Tang, X.; Shi, Y.; Le, G. Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity. *Food Funct.* **2014**, *5*, 1241–1249, doi:10.1039/c3fo60630a.
97. Kemper, J.K.; Xiao, Z.; Ponugoti, B.; Miao, J.; Fang, S.; Kanamaluru, D.; Tsang, S.; Wu, S.Y.; Chiang, C.M.; Veenstra, T.D. FXR acetylation is normally dynamically regulated by p300 and SIRT1 but constitutively elevated in metabolic disease states. *Cell Metab.* **2009**, *10*, 392–404, doi:10.1016/j.cmet.2009.09.009.
98. Chen, M.L.; Yi, L.; Zhang, Y.; Zhou, X.; Ran, L.; Yang, J.; Zhu, J.D.; Zhang, Q.Y.; Mi, M.T. Resveratrol Attenuates Trimethylamine-N-Oxide (TMAO)-Induced Atherosclerosis by Regulating TMAO Synthesis and Bile Acid Metabolism via Remodeling of the Gut Microbiota. *MBio* **2016**, *7*, e02210–e02215, doi:10.1128/mBio.02210-15.
99. Fujita, H.; Yamagami, T. Antihypercholesterolemic effect of Chinese black tea extract in human subjects with borderline hypercholesterolemia. *Nutr. Res.* **2008**, *28*, 450–456, doi:10.1016/j.nutres.2008.04.005.
100. Zhang, H.M.; Wang, C.F.; Shen, S.M.; Wang, G.L.; Liu, P.; Liu, Z.M.; Wang, Y.Y.; Du, S.S.; Liu, Z.L.; Deng, Z.W. Antioxidant phenolic compounds from Pu-erh tea. *Molecules* **2012**, *17*, 14037–14045, doi:10.3390/molecules171214037.

101. Fujita, H.; Yamagami, T. Efficacy and safety of Chinese black tea (Pu-Ehr) extract in healthy and hypercholesterolemic subjects. *Ann. Nutr. Metab.* **2008**, *53*, 33–42, doi:10.1159/000153006.
102. Fujita, H.; Yamagami, T. Extract of black tea (pu-ehr) inhibits postprandial rise in serum cholesterol in mice, and with long term use reduces serum cholesterol and low density lipoprotein levels and renal fat weight in rats. *Phytother. Res. PTR* **2008**, *22*, 1275–1281, doi:10.1002/ptr.2477.
103. Tan, Z.; Luo, M.; Yang, J.; Cheng, Y.; Huang, J.; Lu, C.; Song, D.; Ye, M.; Dai, M.; Gonzalez, F.J.; et al. Chlorogenic acid inhibits cholestatic liver injury induced by alpha-naphthylisothiocyanate: Involvement of STAT3 and NFkappaB signalling regulation. *J. Pharm. Pharmacol.* **2016**, *68*, 1203–1213, doi:10.1111/jphp.12592.
104. Spiller, G.A.; Story, J.A.; Furumoto, E.J.; Chezem, J.C.; Spiller, M. Effect of tartaric acid and dietary fibre from sun-dried raisins on colonic function and on bile acid and volatile fatty acid excretion in healthy adults. *Br. J. Nutr.* **2003**, *90*, 803–807.
105. Spiller, G.A.; Story, J.A.; Lodics, T.A.; Pollack, M.; Monyan, S.; Butterfield, G.; Spiller, M. Effect of sun-dried raisins on bile acid excretion, intestinal transit time, and fecal weight: A dose-response study. *J. Med. Food* **2003**, *6*, 87–91, doi:10.1089/109662003322233477.
106. Williamson, G.; Carughi, A. Polyphenol content and health benefits of raisins. *Nutr. Res.* **2010**, *30*, 511–519, doi:10.1016/j.nutres.2010.07.005.
107. Tebib, K.; Besancon, P.; Rouanet, J.M. Dietary grape seed tannins affect lipoproteins, lipoprotein lipases and tissue lipids in rats fed hypercholesterolemic diets. *J. Nutr.* **1994**, *124*, 2451–2457.
108. Camire, M.E.; Dougherty, M.P. Raisin dietary fiber composition and in vitro bile acid binding. *J. Agric. Food Chem.* **2003**, *51*, 834–837, doi:10.1021/jf025923n.
109. Ngamukote, S.; Makynen, K.; Thilawech, T.; Adisakwattana, S. Cholesterol-lowering activity of the major polyphenols in grape seed. *Molecules* **2011**, *16*, 5054–5061, doi:10.3390/molecules16065054.
110. Yao, R.; Yasuoka, A.; Kamei, A.; Ushiama, S.; Kitagawa, Y.; Rogi, T.; Shibata, H.; Abe, K.; Misaka, T. Nuclear receptor-mediated alleviation of alcoholic fatty liver by polyphenols contained in alcoholic beverages. *PLoS ONE* **2014**, *9*, e87142, doi:10.1371/journal.pone.0087142.
111. Jiao, R.; Zhang, Z.; Yu, H.; Huang, Y.; Chen, Z.Y. Hypocholesterolemic activity of grape seed proanthocyanidin is mediated by enhancement of bile acid excretion and up-regulation of CYP7A1. *J. Nutr. Biochem.* **2010**, *21*, 1134–1139, doi:10.1016/j.jnutbio.2009.10.007.
112. Downing, L.E.; Edgar, D.; Ellison, P.A.; Ricketts, M.L. Mechanistic insight into nuclear receptor-mediated regulation of bile acid metabolism and lipid homeostasis by grape seed procyanidin extract (GSPE). *Cell Biochem. Funct.* **2017**, *35*, 12–32, doi:10.1002/cbf.3247.
113. Del Bas, J.M.; Fernandez-Larrea, J.; Blay, M.; Ardevol, A.; Salvado, M.J.; Arola, L.; Blade, C. Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **2005**, *19*, 479–481, doi:10.1096/fj.04-3095fje.
114. Sembries, S.; Dongowski, G.; Mehrlander, K.; Will, F.; Dietrich, H. Physiological effects of extraction juices from apple, grape, and red beet pomaces in rats. *J. Agric. Food Chem.* **2006**, *54*, 10269–10280, doi:10.1021/jf0618168.
115. Heidker, R.M.; Caiozzi, G.C.; Ricketts, M.L. Dietary procyanidins selectively modulate intestinal farnesoid X receptor-regulated gene expression to alter enterohepatic bile acid recirculation: Elucidation of a novel mechanism to reduce triglyceridemia. *Mol. Nutr. Food Res.* **2016**, *60*, 727–736, doi:10.1002/mnfr.201500795.
116. Shin, M.J.; Cho, Y.; Moon, J.; Jeon, H.J.; Lee, S.M.; Chung, J.H. Hypocholesterolemic effect of daily fisetin supplementation in high fat fed Sprague-Dawley rats. *Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc.* **2013**, *57*, 84–90, doi:10.1016/j.fct.2013.03.010.
117. Del Bas, J.M.; Crescenti, A.; Arola-Arnal, A.; Oms-Oliu, G.; Arola, L.; Caimari, A. Intake of grape procyanidins during gestation and lactation impairs reverse cholesterol transport and increases atherogenic risk indexes in adult offspring. *J. Nutr. Biochem.* **2015**, *26*, 1670–1677, doi:10.1016/j.jnutbio.2015.08.015.
118. Oike, H.; Kobori, M. Resveratrol regulates circadian clock genes in Rat-1 fibroblast cells. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 3038–3040, doi:10.1271/bbb.80426.

119. Miranda, J.; Portillo, M.P.; Madrid, J.A.; Arias, N.; Macarulla, M.T.; Garaulet, M. Effects of resveratrol on changes induced by high-fat feeding on clock genes in rats. *Br. J. Nutr.* **2013**, *110*, 1421–1428, doi:10.1017/s0007114513000755.
120. Baselga-Escudero, L.; Blade, C.; Ribas-Latre, A.; Casanova, E.; Salvado, M.J.; Arola, L.; Arola-Arnal, A. Grape seed proanthocyanidins repress the hepatic lipid regulators miR-33 and miR-122 in rats. *Mol. Nutr. Food Res.* **2012**, *56*, 1636–1646, doi:10.1002/mnfr.201200237.
121. Baselga-Escudero, L.; Blade, C.; Ribas-Latre, A.; Casanova, E.; Suarez, M.; Torres, J.L.; Salvado, M.J.; Arola, L.; Arola-Arnal, A. Resveratrol and EGCG bind directly and distinctively to miR-33a and miR-122 and modulate divergently their levels in hepatic cells. *Nucleic Acids Res.* **2014**, *42*, 882–892, doi:10.1093/nar/gkt1011.
122. Jahanafrooz, Z.; Motamed, N.; Bakhshandeh, B. Effects of miR-21 downregulation and silibinin treatment in breast cancer cell lines. *Cytotechnology* **2017**, doi:10.1007/s10616-017-0076-5.
123. Su, X.; Zhang, J.; Wang, H.; Xu, J.; He, J.; Liu, L.; Zhang, T.; Chen, R.; Kang, J. Phenolic Acid Profiling, Antioxidant, and Anti-Inflammatory Activities, and miRNA Regulation in the Polyphenols of 16 Blueberry Samples from China. *Molecules* **2017**, *22*, doi:10.3390/molecules22020312.
124. Milenkovic, D.; Deval, C.; Gouranton, E.; Landrier, J.F.; Scalbert, A.; Morand, C.; Mazur, A. Modulation of miRNA expression by dietary polyphenols in apoE deficient mice: A new mechanism of the action of polyphenols. *PLoS ONE* **2012**, *7*, e29837, doi:10.1371/journal.pone.0029837.
125. Shimoyama, M.D.P.J.; Hayman, G.T.; Laulederkind, S.J.; Liu, W.; Nigam, R.; Petri, V.; Smith, J.R.; Tutaj, M.; Wang, S.J.; Worthey, E.; et al. The Rat Genome Database 2015: Genomic, phenotypic and environmental variations and disease. *Nucleic Acids Res*. **2014**, *43*, D743–D750.
126. Arola-Arnal, A.; Blade, C. Proanthocyanidins modulate microRNA expression in human HepG2 cells. *PLoS ONE* **2011**, *6*, e25982, doi:10.1371/journal.pone.0025982.
127. Rao, A.; Kosters, A.; Mells, J.E.; Zhang, W.; Setchell, K.D.; Amanso, A.M.; Wynn, G.M.; Xu, T.; Keller, B.T.; Yin, H.; et al. Inhibition of ileal bile acid uptake protects against nonalcoholic fatty liver disease in high-fat diet-fed mice. *Sci. Transl. Med.* **2016**, *8*, 357ra122, doi:10.1126/scitranslmed.aaf4823.
128. Bhat, B.G.; Rapp, S.R.; Beaudry, J.A.; Napawan, N.; Butteiger, D.N.; Hall, K.A.; Null, C.L.; Luo, Y.; Keller, B.T. Inhibition of ileal bile acid transport and reduced atherosclerosis in apoE-/-mice by SC-435. *J. Lipid Res.* **2003**, *44*, 1614–1621, doi:10.1194/jlr.M200469-JLR200.
129. Huff, M.W.; Telford, D.E.; Edwards, J.Y.; Burnett, J.R.; Barrett, P.H.R.; Rapp, S.R.; Napawan, N.; Keller, B.T. Inhibition of the Apical Sodium-Dependent Bile Acid Transporter Reduces LDL Cholesterol and ApoB by Enhanced Plasma Clearance of LDL ApoB. *Arterioscler. Thromb. Vasc. Biol.* **2002**, *22*, 1884–1891, doi:10.1161/01.atv.0000035390.87288.26.
130. Thomas, C.; Landrier, J.F.; Gaillard, D.; Grober, J.; Monnot, M.C.; Athias, A.; Besnard, P. Cholesterol dependent downregulation of mouse and human apical sodium dependent bile acid transporter (ASBT) gene expression: Molecular mechanism and physiological consequences. *Gut* **2006**, *55*, 1321–1331, doi:10.1136/gut.2005.085555.
131. Alrefai, W.A.; Sarwar, Z.; Tyagi, S.; Saksena, S.; Dudeja, P.K.; Gill, R.K. Cholesterol modulates human intestinal sodium-dependent bile acid transporter. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2005**, *288*, G978–G985, doi:10.1152/ajpgi.00379.2004.
132. Xu, G.; Shneider, B.L.; Shefer, S.; Nguyen, L.B.; Batta, A.K.; Tint, G.S.; Arrese, M.; Thevananther, S.; Ma, L.; Stengelin, S.; et al. Ileal bile acid transport regulates bile acid pool, synthesis, and plasma cholesterol levels differently in cholesterol-fed rats and rabbits. *J. Lipid Res.* **2000**, *41*, 298–304.
133. Torchia, E.C.; Cheema, S.K.; Agellon, L.B. Coordinate regulation of bile acid biosynthetic and recovery pathways. *Biochem. Biophys. Res. Commun.* **1996**, *225*, 128–133, doi:10.1006/bbrc.1996.1141.
134. Zhang, Y.K.; Guo, G.L.; Klaassen, C.D. Diurnal variations of mouse plasma and hepatic bile acid concentrations as well as expression of biosynthetic enzymes and transporters. *PLoS ONE* **2011**, *6*, e16683, doi:10.1371/journal.pone.0016683.

135. Odermatt, A.; Da Cunha, T.; Penno, C.A.; Chandsawangbhuwana, C.; Reichert, C.; Wolf, A.; Dong, M.; Baker, M.E. Hepatic reduction of the secondary bile acid 7-oxolithocholic acid is mediated by 11beta-hydroxysteroid dehydrogenase 1. *Biochem. J.* **2011**, *436*, 621–629, doi:10.1042/bj20110022.
136. Jones, B.V.; Begley, M.; Hill, C.; Gahan, C.G.M.; Marchesi, J.R. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13580–13585, doi:10.1073/pnas.0804437105.
137. Sayin, S.I.; Wahlstrom, A.; Felin, J.; Jantti, S.; Marschall, H.U.; Bamberg, K.; Angelin, B.; Hyotylainen, T.; Oresic, M.; Backhed, F. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metab.* **2013**, *17*, 225–235, doi:10.1016/j.cmet.2013.01.003.
138. Wostmann, B.S. Intestinal bile acids and cholesterol absorption in the germfree rat. *J. Nutr.* **1973**, *103*, 982–990.
139. Kellogg, T.F.; Wostmann, B.S. Fecal neutral steroids and bile acids from germfree rats. *J. Lipid Res.* **1969**, *10*, 495–503.
140. Madsen, D.; Beaver, M.; Chang, L.; Bruckner-Kardoss, E.; Wostmann, B. Analysis of bile acids in conventional and germfree rats. *J. Lipid Res.* **1976**, *17*, 107–111.
141. Li, F.; Jiang, C.; Krausz, K.W.; Li, Y.; Albert, I.; Hao, H.; Fabre, K.M.; Mitchell, J.B.; Patterson, A.D.; Gonzalez, F.J. Microbiome remodelling leads to inhibition of intestinal farnesoid X receptor signalling and decreased obesity. *Nat. Commun.* **2013**, *4*, 2384, doi:10.1038/ncomms3384.
142. Jeun, J.; Kim, S.; Cho, S.Y.; Jun, H.J.; Park, H.J.; Seo, J.G.; Chung, M.J.; Lee, S.J. Hypocholesterolemic effects of Lactobacillus plantarum KCTC3928 by increased bile acid excretion in C57BL/6 mice. *Nutrition* **2010**, *26*, 321–330, doi:10.1016/j.nut.2009.04.011.
143. Jiang, C.; Xie, C.; Lv, Y.; Li, J.; Krausz, K.W.; Shi, J.; Brocker, C.N.; Desai, D.; Amin, S.G.; Bisson, W.H.; et al. Intestine-selective farnesoid X receptor inhibition improves obesity-related metabolic dysfunction. *Nat. Commun.* **2015**, *6*, 10166, doi:10.1038/ncomms10166.
144. Jones, M.L.; Martoni, C.J.; Parent, M.; Prakash, S. Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active Lactobacillus reuteri NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. *Br. J. Nutr.* **2012**, *107*, 1505–1513, doi:10.1017/s0007114511004703.
145. Jones, M.L.; Martoni, C.J.; Prakash, S. Cholesterol lowering and inhibition of sterol absorption by Lactobacillus reuteri NCIMB 30242: A randomized controlled trial. *Eur. J. Clin. Nutr.* **2012**, *66*, 1234–1241, doi:10.1038/ejcn.2012.126.
146. De Smet, I.; De Boever, P.; Verstraete, W. Cholesterol lowering in pigs through enhanced bacterial bile salt hydrolase activity. *Br. J. Nutr.* **1998**, *79*, 185–194.
147. Espín, J.C.; González-Sarrías, A.; Tomás-Barberán, F.A. The gut microbiota: A key factor in the therapeutic effects of (poly)phenols. *Biochem. Pharmacol.* **2017**, doi:10.1016/j.bcp.2017.04.033.
148. Lee, H.C.; Jenner, A.M.; Low, C.S.; Lee, Y.K. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Res. Microbiol.* **2006**, *157*, 876–884, doi:10.1016/j.resmic.2006.07.004.
149. Nakamura, Y.; Kaihara, A.; Yoshii, K.; Tsumura, Y.; Ishimitsu, S.; Tonogai, Y. Effects of the Oral Administration of Green Tea Polyphenol and Tannic Acid on Serum and Hepatic Lipid Contents and Fecal Steroid Excretion in Rats. *J. Health Sci.* **2001**, *47*, 107–117, doi:10.1248/jhs.47.107.
150. Han, Y.; Haraguchi, T.; Iwanaga, S.; Tomotake, H.; Okazaki, Y.; Mineo, S.; Moriyama, A.; Inoue, J.; Kato, N. Consumption of some polyphenols reduces fecal deoxycholic acid and lithocholic acid, the secondary bile acids of risk factors of colon cancer. *J. Agric. Food Chem.* **2009**, *57*, 8587–8590, doi:10.1021/jf900393k.
151. Tzounis, X.; Vulevic, J.; Kuhnle, G.G.; George, T.; Leonczak, J.; Gibson, G.R.; Kwik-Uribe, C.; Spencer, J.P. Flavanol monomer-induced changes to the human faecal microflora. *Br. J. Nutr.* **2008**, *99*, 782–792, doi:10.1017/s0007114507853384.
152. Queipo-Ortuno, M.I.; Boto-Ordonez, M.; Murri, M.; Gomez-Zumaquero, J.M.; Clemente-Postigo, M.; Estruch, R.; Cardona Diaz, F.; Andres-Lacueva, C.; Tinahones, F.J. Influence of red wine

polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. *Am. J. Clin. Nutr.* **2012**, *95*, 1323–1334, doi:10.3945/ajcn.111.027847.
153. Louis, P.; Flint, H.J. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* **2009**, *294*, 1–8, doi:10.1111/j.1574-6968.2009.01514.x.
154. Scott, K.P.; Martin, J.C.; Duncan, S.H.; Flint, H.J. Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, in vitro. *Fems Microbiol. Ecol.* **2014**, *87*, 30–40, doi:10.1111/1574-6941.12186.
155. Dolara, P.; Luceri, C.; Filippo, C.D.; Femia, A.P.; Giovannelli, L.; Caderni, G.; Cecchini, C.; Silvi, S.; Orpianesi, C.; Cresci, A. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* **2005**, *591*, 237–246, doi:10.1016/j.mrfmmm.2005.04.022.
156. Caderni, G.; Remy, S.; Cheynier, V.; Morozzi, G.; Dolara, P. Effect of complex polyphenols on colon carcinogenesis. *Eur. J. Nutr.* **1999**, *38*, 126–132, doi:10.1007/s003940050053.
157. Vendrame, S.; Guglielmetti, S.; Riso, P.; Arioli, S.; Klimis-Zacas, D.; Porrini, M. Six-week consumption of a wild blueberry powder drink increases bifidobacteria in the human gut. *J. Agric. Food Chem.* **2011**, *59*, 12815–12820, doi:10.1021/jf2028686.
158. Yamakoshi, J.; Tokutake, S.; Kikuchi, M.; Kubota, Y.; Konishi, H.; Mitsuoka, T. Effect of Proanthocyanidin-Rich Extract from Grape Seeds on Human Fecal Flora and Fecal Odor. *Microb. Ecol. Health Dis.* **2001**, *13*, 25–31, doi:10.1080/089106001750071672.
159. Larrosa, M.; Yanez-Gascon, M.J.; Selma, M.V.; Gonzalez-Sarrias, A.; Toti, S.; Ceron, J.J.; Tomas-Barberan, F.; Dolara, P.; Espin, J.C. Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. *J. Agric. Food Chem.* **2009**, *57*, 2211–2220, doi:10.1021/jf803638d.
160. Le Roy, C.I.; Wells, P.M.; Si, J.; Raes, J.; Bell, J.T.; Spector, T.D. Red Wine Consumption Associated With Increased Gut Microbiota alpha-diversity in 3 Independent Cohorts. *Gastroenterology* **2019**, doi:10.1053/j.gastro.2019.08.024.
161. Fotschki, B.; Juskiewicz, J.; Jurgonski, A.; Rigby, N.; Sojka, M.; Kolodziejczyk, K.; Mackie, A.; Zdunczyk, Z. Raspberry pomace alters cecal microbial activity and reduces secondary bile acids in rats fed a high-fat diet. *J. Nutr. Biochem.* **2017**, *46*, 13–20, doi:10.1016/j.jnutbio.2017.03.004.
162. Kim, H.; Kim, D.H.; Seo, K.H.; Chon, J.W.; Nah, S.Y.; Bartley, G.E.; Arvik, T.; Lipson, R.; Yokoyama, W. Modulation of the intestinal microbiota is associated with lower plasma cholesterol and weight gain in hamsters fed chardonnay grape seed flour. *J. Agric. Food Chem.* **2015**, *63*, 1460–1467, doi:10.1021/jf5026373.
163. Ren, K.; Jiang, T.; Zhao, G.J. Quercetin induces the selective uptake of HDL-cholesterol via promoting SR-BI expression and the activation of the PPARgamma/LXRalpha pathway. *Food Funct.* **2018**, *9*, 624–635, doi:10.1039/c7fo01107e.
164. Hou, D.X.; Luo, D.; Tanigawa, S.; Hashimoto, F.; Uto, T.; Masuzaki, S.; Fujii, M.; Sakata, Y. Prodelphinidin B-4 3'-O-gallate, a tea polyphenol, is involved in the inhibition of COX-2 and iNOS via the downregulation of TAK1-NIF-kappa B pathway. *Biochem. Pharmacol.* **2007**, *74*, 742–751, doi:10.1016/j.bcp.2007.06.006.
165. Crestani, M.; Galli, G.; Chiang, J.Y. Genomic cloning, sequencing, and analysis of the hamster cholesterol 7 alpha-hydroxylase gene (CYP7). *Arch. Biochem. Biophys.* **1993**, *306*, 451–460, doi:10.1006/abbi.1993.1537.
166. Ramirez, M.I.; Karaoglu, D.; Haro, D.; Barillas, C.; Bashirzadeh, R.; Gil, G. Cholesterol and bile acids regulate cholesterol 7 alpha-hydroxylase expression at the transcriptional level in culture and in transgenic mice. *Mol. Cell. Biol.* **1994**, *14*, 2809–2821.
167. Gatmaitan, Z.; Jefferson, D.M.; Ruiz-Opazo, N.; Biempica, L.; Arias, I.M.; Dudas, G.; Leinwand, L.A.; Reid, L.M. Regulation of growth and differentiation of a rat hepatoma cell line by the synergistic interactions of hormones and collagenous substrata. *J. Cell Biol.* **1983**, *97*, 1179–1190.

168. Enat, R.; Jefferson, D.M.; Ruiz-Opazo, N.; Gatmaitan, Z.; Leinwand, L.A.; Reid, L.M. Hepatocyte proliferation in vitro: Its dependence on the use of serum-free hormonally defined medium and substrata of extracellular matrix. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 1411–1415.
169. Davalos, A.; Fernandez-Hernando, C.; Cerrato, F.; Martinez-Botas, J.; Gomez-Coronado, D.; Gomez-Cordoves, C.; Lasuncion, M.A. Red grape juice polyphenols alter cholesterol homeostasis and increase LDL-receptor activity in human cells in vitro. *J. Nutr.* **2006**, *136*, 1766–1773.