SPECIAL ARTICLE

Dietary flavonoids and nitrate: effects on nitric oxide and vascular function.

Catherine P Bondonno PhD, Kevin D Croft PhD, Natalie Ward PhD,

Michael J Considine PhD, Jonathan M Hodgson PhD

CP Bondonno, KD Croft, NC Ward, JM Hodgson:

School of Medicine and Pharmacology, University of Western Australia, Perth, Western Australia 6000, Australia

MJ Considine:

School of Plant Biology, University of Western Australia, Perth, Western Australia 6000, Australia

Department of Agriculture and Food Western Australia, South Perth, Western Australia 6000, Australia

Address for correspondence and requests for reprints:

Catherine P Bondonno

School of Medicine and Pharmacology

Level 3, Medical Research Foundation

Rear 50 Murray St, Perth Western Australia, Australia WA6000

GPO Box X2213 Perth Western Australia, Australia WA6847

Tel: 618 9224 0342

Fax: 618 9224 0246

Email: cbondonno@meddent.uwa.edu.au

ABSTRACT

Emerging evidence highlights dietary flavonoids and nitrate as candidates that may explain at least part of the cardioprotective effect of a fruit and vegetable diet. Nitric oxide (NO) plays a pivotal role in cardiovascular health. Components of a fruit and vegetable diet that are cardioprotective in part through effects on NO status could substantially reduce the cardiovascular risk profile of the general population with increased intake. Epidemiological evidence suggests that dietary flavonoids and nitrate have a cardioprotective effect. Clinical trials with flavonoid and nitrate rich foods have observed benefits on measures of vascular health. While the molecular mechanisms by which flavonoids and nitrate are cardioprotective are not completely understood, recent evidence suggests both non-specific and specific effects through NO pathways. It is the purpose of this review to present an overview of NO and its key role in cardiovascular health; and to discuss the possible vascular benefits of flavonoids and nitrate, individually and in combination, through effects on NO status.

Key Words: flavonoids, nitrate, nitric oxide, vascular function

Abbreviations

ADP, adenosine diphosphate; COMT, catechol-O-methyltransferases; CBG, cystolic β – glucosidase; eNOS, endothelial nitric oxide synthase; FMD, flow mediated dilatation; FFQ, food frequency questionnaire; LPH, lactase-phlohizin-hydrolase; MRP, multidrug resistance protein; NO, nitric oxide; NOS, nitric oxide synthase ; P-gp, P-glycoprotein; PKG, cyclic GMP-dependent protein Kinase; PWV, pulse wave velocity; RSNO, *S*-nitrosothiols; SGLT1, sodium-dependent glucose transporter; SULT, sulphotransferases; BH_4, tetrahydrobiopterin; UGTs, uridine-5'-diphosphate glucoronyltransferases

INTRODUCTION

Cardiovascular disease is of mounting scientific and public concern. Worldwide, it is the leading noncommunicable disease, accounting for 30% (17.3 million) deaths annually[1]. For those living with the disease, quality of life is impacted with a significant number having an associated disability. The majority of cardiovascular disease risk factors, such as dyslipidemia, high blood pressure, and obesity may be associated with an unhealthy life style and poor diet. These risk factors are modifiable, highlighting the critical importance of prevention strategies such as healthy eating. A diet rich in fruit and vegetables is significantly associated with a reduction in cardiovascular disease[2-4]. There is, therefore, considerable interest in identifying the cardioprotective components of a fruit and vegetable diet and the corresponding pathways through which they mediate their benefits. With the discovery of nitric oxide (NO) and the pivotal role it plays in endothelial function and therefore cardiovascular health, attention has focused on the components of a fruit and vegetable diet that could mediate their cardioprotective benefits through NO. Currently, among the phytochemicals being actively studied are the flavonoids and nitrate. It is the aim of this article to provide an overview of the role of NO in the cardiovasculature, and to review the role that flavonoids and nitrate, through effects on NO status, may play in cardiovascular health. We address the evidence that flavonoids and nitrate make a significant contribution to the cardiovascular health benefits of a fruit and vegetable-rich diet via effects on NO status.

VASCULAR FUNCTION AND NITRIC OXIDE

Endothelial function and dysfunction

Vital to cardiovascular health is a healthy endothelium, a single layer of cells found between the circulating blood and vascular smooth muscle cells. Originally seen as merely a simple semi permeable barrier between blood and interstitium, the endothelium is now known to

control vascular tone (influencing blood pressure and arterial stiffness), inflammation, permeability and growth as well as blood fluidity and coagulation. The healthy endothelium maintains vascular homeostasis by synthesis or release of a wide range of molecules, including NO, in response to physical and chemical stimuli[5]. Endothelial function could serve as an index of cardiovascular health[5] and a change in endothelial function (dysfunction) is a preliminary (and reversible) step in the development of cardiovascular disease [6,7].

Endothelial dysfunction is defined as an impairment of endothelium-dependent relaxation, with a tendency towards a proinflammatory and prothrombotic state[8]. Impaired endothelial function is associated with a higher risk of cardiovascular events [9] and endothelial dysfunction is implicated in cardiovascular pathologies such as hypertension[10], atherosclerosis[11] and stroke[12]. Obesity, smoking, aging, hypercholesterolaemia, hypertension, hyperglycaemia, systemic infection and a family history of early atherosclerotic disease, all cardiovascular risk factors, are strongly associated with endothelial dysfunction[5]. Improvements in endothelial function could be partly responsible for the reduction in cardiovascular risk observed with interventions such as exercise, smoking cessation, weight loss, certain medications and healthy diet[5,13].

While the mechanisms behind healthy endothelial function and the development of endothelial dysfunction are complex, a molecule playing a central role in both is NO.

The endothelial L-arginine NO Synthase Pathway

NO is generated via the L-arginine NO synthase pathway. This pathway is well defined and has been reviewed in depth[14-18]. The production of NO from the amino acid L-arginine is catalysed by a family of nitric oxide synthase (NOS) enzymes. Endothelial nitric oxide synthase (eNOS), constitutively expressed by endothelial cells, is the main source of NO in the vasculature. eNOS synthesis is stimulated by both mechanical (shear stress and cyclic strain) and biochemical stimuli (thrombin, adenosine diphosphate (ADP), serotonin,

acetylcholine and bradykinin). In response to these stimuli, Ca^{2+} is released from intracellular stores which then binds to calmodulin forming a Ca^{2+}-calmodulin complex, essential for activating eNOS [17]. eNOS catalyses the synthesis of NO by the 5-electron oxidation of the terminal guanidine nitrogen atom of L-arginine with nicotinamide adenine dinucluotide phosphate (NADPH) and molecular oxygen as co-substrates. L-citrulline is produced as a by-product. eNOS activity is tightly controlled by cofactors such as flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin (BH_4), calmodulin and heme[19].

NO is an extremely labile molecule with a very short half-life of 1-2 s. Following its synthesis in endothelial cells, most of the NO diffuses across the endothelial cell membrane into the adjacent vascular smooth muscle cells. Here it activates guanylate cyclase by binding to its heme moiety, causing a rise in cGMP concentrations. cGMP, through its principal mediator Protein Kinase G (PKG), is responsible for many of the biological effects of NO[20].

A significant proportion of the NO that does not cross the endothelial cell membrane to activate cGMP will react with both oxyhaemoglobin (forming nitrate and methaemoglobin) and deoxyhaemoglobin (resulting in iron-nitrosyl haemoglobin)[21]. NO also reacts rapidly with superoxide to form peroxynitrite, a reaction thought to be responsible for the pathological effects associated with NO[22]. The small amount of NO that escapes inactivation by molecules such as haemoglobin and superoxide, approximately 20%, is either oxidised to nitrite, reacts with thiol groups on proteins to form *S*-nitrosothiols (RSNOs) or reacts with lipids to form nitrated lipids[23].

RSNOs, nitrite and nitrate are important end products of NO metabolism. It is now established that they preserve NO from local inactivation, transport NO throughout the body and become a storage pool of NO. RSNOs, nitrite and nitrate via conversion to nitrite, have the potential to be converted back to NO, which may explain some of the systemic effects of NO that occur after its administration by inhalation or infusion[24-28]. Rassaf et al have

demonstrated that when NO is administered intravenously, the systemic effects observed are mediated by the conversion of NO to RSNOs[29].

Nitrate-nitrite-NO pathway

An alternate source of NO is the recently described nitrate-nitrite-NO pathway[30-32]. This pathway and the L-arginine-NOS pathway described above are linked. Plasma nitrate derives from nitrate formed as an end product of NO metabolism as well as nitrate obtained from the diet (obtained primarily from green leafy vegetables and beetroot). In the enterosalivary nitrate-nitrite-NO pathway about 25% of plasma nitrate is actively absorbed by the salivary glands and concentrated in the saliva[33]. In the oral cavity facultative anaerobic nitrate-reducing bacteria, located in the deep clefts on the dorsal surface of the tongue, reduce nitrate to nitrite[34]. In the acidic environment of the stomach some salivary nitrite is reduced to NO with localised gastrointestinal benefits[35-37]. The remaining nitrite is absorbed into the blood stream[38] and, with nitrite formed as a by-product of NO metabolism, becomes a source of NO[38,39].

Vascular effects of NO

Endothelial derived NO plays a key role in maintaining vascular homeostasis and integrity. A number of cardiovascular disorders including hypertension, atherosclerosis and ischaemic disease are associated with endothelial dysfunction and a reduction in production and/or bioavailability of NO. Whether decreased NO status is the cause or result of endothelial dysfunction is not yet understood. Accepted, however, is the key role of NO in maintaining vascular tone, as well as the importance of its antithrombotic, antiatherogenic and antiproliferative properties[17].

Basal vascular tone is achieved from a balance between NO, vasoconstrictors and the sympathetic nervous system. Low levels of NO are continuously released in the

endothelium[40] and through the cGMP mediated relaxation of vascular smooth muscle cells, NO maintains vasorelaxation. This basal release of NO plays an important role in regulation of blood pressure and blood flow[40,41]. Indeed, increased local arterial stiffness, elevated blood pressure as well as reduced blood flow are observed after inhibition of NO synthesis[42-44].

NO maintains vascular integrity by suppressing platelet aggregation, leukocyte migration, cellular adhesion to the endothelium as well as vascular smooth muscle cell proliferation[45]. These antithrombotic, antiatherogenic, antiinflammatory and antiproliferative properties of NO play an important role in cardiovascular health. Indeed, decreased production and/or bioavailability of NO could promote a vascular phenotype more susceptible to atherogenesis. This has been demonstrated experimentally in animals by inhibiting its synthesis[46]. In addition, mice lacking eNOS have endothelial dysfunction, are hypertensive and show a more severe outcome in response to vascular injury, stroke, cerebral ischemia and diet induced atherosclerosis[18].

FLAVONOIDS AND VASCULAR FUNCTION

Findings from epidemiological studies[47-49], that increased fruit and vegetable intake could reduce the incidence of cardiovascular disease have sparked wide research to determine which phytochemicals and which mechanisms are responsible. For many years this effect was considered to be due to the antioxidant properties of plant constituents such as the vitamins and carotenoids. When a number of large studies failed to confirm this hypothesis[50,51], attention focused on other potentially bioactive compounds as well as other protective mechanisms. Currently, among the phytochemicals being actively studied as potential candidates for the cardioprotective effects of fruit and vegetables are a large group of phytochemicals, the polyphenols.

In the last decade, polyphenols have become one of the most extensively studied groups of nutritional molecules. Polyphenols are produced as secondary plant metabolites and are found

in great abundance in our diet. More than 8000 phenolic compounds have been identified and are divided into groups according to chemical structure characterised by the presence of one or more phenolic rings (**Figure 1**). They can be classed as flavonoid and non-flavonoid compounds. Flavonoids are the largest subclass of polyphenols with quercetin and (-)-epicatechin being among the most studied of the individual flavonoids.

Structure of flavonoids

Structurally, flavonoids have a common C6-C3-C6 structure consisting of 2 aromatic rings (A and B rings) that are linked by a 3-carbon bridge (C-ring) forming an oxygenated heterocycle. Subclasses of the flavonoids, namely the flavonols, flavones, flavan-3-ols (including the proanthocyanidins which are primarily flavan-3-ol polymers), flavanones, anthocyanins and isoflavones, are defined by differences in the oxidation state and functional groups of the C-ring as well as by the connection of the B- to the C- ring (**Table 1**[52,53]). In addition within each subclass is the potential for substitution, following metabolism, in the A and B rings with phenolic hydroxyls, O-sugars, methoxy groups, sulphates and glucoronides[54]. This contributes to their structural complexity with, to date, more than 6000 individual flavonoid molecules identified[55]. Most flavonoid subclasses occur naturally as glycosides (bound to one or more sugar molecules) and other conjugates with the exception of flavan-3-ols which tend to exist as aglycones (not bound to a sugar molecule).

Dietary Sources

Table 1 summarises the typical food sources of the flavonoid subclasses. Estimates of total flavonoid consumption vary: 20->70 mg/d[52]; 65-250 mg/d[56] and 1g/d[57] partly due to the number of different flavonoid subclasses considered. Intake is difficult to assess, as although flavonoids are ubiquitous in foods of plant origin, their distribution is not uniform. In addition, the level of flavonoids in a given food is dependent on the cultivar/variety, agricultural methods, growth environment, time of harvest, method of harvesting, storage

198 conditions as well as post-harvest processing and cooking methods[52,58]. The normal dietary
199 intake of particular cultures determines which foods and consequently which flavonoid
200 subclasses are consumed. The primary dietary supply of flavonoids in western society
201 includes tea, red wine, chocolate, cocoa, fruit, vegetables and legumes while in Japan and
202 Indonesia, soy and soy foods are highly consumed, which provides high levels of isoflavones
203 in the diet. There is also evidence to suggest that intake of certain subclasses and specific
204 flavonoids may be more important than total flavonoid intake with regard to potential health
205 benefits. Additionally, there is a wide variation in the bioavailability of flavonoid subclasses
206 with some reaching higher biologically active concentrations than others[53,58].

207 **Absorption, metabolism and bioavailability**

208 Absorption, metabolism and bioavailability are remarkably different between the flavonoid
209 subclasses. Indeed, the most abundant flavonoids in our diet may not necessarily be the most
210 bioavailable or the most biologically active[59]. Flavonoid subclasses have different absorption
211 kinetics and are highly metabolised with the resulting metabolites differing in biological
212 activity from their parent compound and from each other. Establishing the absorption kinetics,
213 metabolism and identity of flavonoid metabolites is now recognised to be imperative in
214 solving the mechanisms by which dietary flavonoids may exert beneficial effects on health.
215 This is an active area of research and is summarised in **Figure 2** depicting quercetin
216 glycosides (flavonols) and (-)-epicatechin (flavan-3-ol) as examples.

217 The majority of flavonoids occur naturally as glycosides apart from the flavan-3-ols, which
218 exist as aglycones. The absorption kinetics of the flavonoid glycosides, therefore, is quite
219 different to that of flavan-3-ols. Absorption of flavonoid glycosides first requires the release
220 of the aglycone by hydrolysis. This process typically occurs in the small intestine[60] or later in
221 the colon. In the small intestine, hydrolysis occurs by action of enteric membrane bound
222 lactase-phlohizin-hydrolase (LPH) or cystolic β –glucosidase (CBG) within the epithelial

cells. LPH has a broad affinity for flavonoid-O- β-D-glucosides. The released aglycone has the potential to enter epithelial cells by possibly passive diffusion ("LPH/diffusion")[61]. The action of CBG requires active transport into the epithelial cells and it is thought that the active sodium-dependent glucose transporter (SGLT1) is involved ("SGLT/CBG")[62]. Results of recent studies, however, seem to indicate that the "transport/CBG" pathway of flavonoid absorption may not exist[63]. Flavonoid glycosides not absorbed in the small intestine pass into the colon where they are acted on by enterobacterial β –glucosidases. The type and position of the glycoside moiety affects the affinity of the hydrolysing enzymes[64] and, therefore, also determines site of absorption. For example quercetin 4'-glucoside peaks 0.5-0.7 h after ingestion whereas quercetin -3β-rutinoside, with an attenuated bioavailability in comparison, peaks at 6-9 h[65,66]. This indicates that the quercetin glucosides can be absorbed in the small intestine whereas those attached to a rhamnose require hydrolysis by colon microflora before limited absorption in the colon can take place. In contrast to the flavonoid glycosides, the flavan-3-ol aglycones occur as monomers, oligomers and polymers. The monomers are absorbed in the small intestine in a process influenced by steroechemical configuration. For example, absorption of (-)-epicatechin is greater than (+)-epicatechin and (+)-catechin is greater than (-)-catechin[67]. Flavan-3-ol oligomers with a degree of polymerisation (DP) less than 2 may be absorbed from the small intestine but to a much lower extent than the monomers[68]. Oligomers with a DP greater than 2[68] and larger molecular weight proanthocyanidins are not absorbed and reach the colon where they are degraded by microflora to phenolic acids[69]. A small proportion of these phenolic acids can be absorbed and may have bioactivity *in vivo*.

It is well established that flavonoids undergo extensive metabolism after ingestion[53,64]. After the release of the aglycone by hydrolysis, glucuronidation, sulphation and methylation of the aglycones occurs in the intestinal epithelial cells by the action of sulphotransferases (SULT),

uridine-5'-diphosphate glucoronyltransferases (UGTs) and catechol-O-methyltransferases (COMT) before they enter the blood stream[70]. There is some efflux of these metabolites back into the small intestine, which could involve the multidrug resistance protein (MRP) and P-glycoprotein (P-gp). This conjugation process is very efficient with no aglycones, apart from the catechins, measured in plasma and urine[54]. Once in the blood stream, the conjugates rapidly reach the liver where they are further methylated, glucoronidated, or sulphated as part of phase II liver metabolism[71]. Metabolism can also occur in the kidney. Excretion is via the urine or the conjugates are recycled back to the small intestine through the bile. Flavonoids not absorbed in the small intestine together with hepatic metabolites secreted with bile (enetero-hepatic circulation) will be degraded in the colon by colonic microflora[72]. The colon contains a diverse microbial population of obligate anaerobes and facultative anaerobes. Bacterial enzymes cleave conjugating compounds with aglycones appearing briefly, and can break down the flavonoid ring structure resulting in smaller molecules that include phenolic acids and hydroxycinnamates[72]. After absorption, these molecules may be further metabolised by the liver before excretion.

The bioavailability of flavonoids is dependent on the absorption kinetics and degree of metabolism of the flavonoid subclasses, as described above. Bioavailability is also dependent on the composition of the diet and the food matrix[73], tissue distribution as well as host differences such as physiological state, genetic polymorphisms of genes involved in absorption, metabolism or elimination, and variations (both inter and intra) in intestinal microflora[74]. A large amount of unidentified metabolites may be present in the plasma[75]. Future metabolomic studies and new mass spectrometry based analytical methods have the potential to identify these metabolites and decipher the complex relationship between flavonoid metabolites, factors influencing their bioavailability and their biological effects[76].

Beneficial effects on vascular health

Epidemiological studies suggest an inverse association between flavonoid consumption and the risk of cardiovascular disease. Cardiovascular protection by flavonoid rich foods could occur through effects on blood pressure, endothelial function and platelet reactivity. Results of studies with flavonoid rich foods and their cardiovascular effects are, however, inconsistent and the molecular mechanisms involved are not completely understood. This could be due, in part, to the fact that food matrices contain a large number of flavonoids, as well as other phytochemicals, with unequal physiological effects. There is also a potential for inhibition, additive effects or synergism among phytochemicals. Studies with flavonoid rich foods attribute the benefits observed to the flavonoids present. Few human trials have been performed with isolated flavonoids. Studies with quercetin and (-)-epicatechin, indicate that these flavonoids could be among the key players in the cardioprotective effects mediated by flavonoids.

Epidemiological evidence

Epidemiological studies have examined the relationship between risk of cardiovascular disease with total flavonoid intake, individual flavonoid subclasses and foods rich in flavonoids. While overall the evidence suggests a protective effect, there are contradictory findings, which could be due to a number of study limitations.

The association between total flavonoid intake and reduced cardiovascular disease risk has been both supported and questioned by epidemiological studies. In 2005, Arts and Hollman evaluated the outcomes of 12 cohort studies examining flavonoid intake and coronary artery disease as well as 5 cohort studies on flavonoid intake and risk of stroke[77]. Since this publication, 4 additional prospective studies have examined total flavonoid intake and cardiovascular disease mortality[78-81]. Only five out of the 15 studies found a significant reduction in cardiovascular disease risk after multivariate adjustment with high flavonoid

297 intake compared to low flavonoid intake[77,78]. However, when individual flavonoid subclasses
298 are examined, a protective effect is observed for certain subclasses[78].

299 The fact that certain flavonoid subclasses could be more cardioprotective than others is not
300 surprising given their diversity in structure, metabolism and bioactivity. Two flavonoid
301 subclasses that are consistently examined and associated with reduced risk in epidemiological
302 studies are the flavonols (predominantly quercetin) and the flavones[77,82]. A meta-analysis
303 examining the association between flavonol intake and coronary heart disease mortality
304 observed a 20% reduction in risk for men and woman in the highest tertile of flavonol intake
305 compared to those in the lowest tertile[83]. Additionally, an independent meta-analysis observed
306 a 20% reduction in risk of stroke for high flavonol intake compared to low intake [84]. Evidence
307 for an association between other flavonoid subclasses and reduced cardiovascular risk is
308 limited[82], as fewer studies have examined this association. No meta-analyses on the other
309 flavonoid subclasses and risk of cardiovascular disease have been performed to date.

310 A large number of epidemiological studies have examined the relationship between flavonoid-
311 rich foods and cardiovascular disease. Positive associations with reduced risk have been
312 observed for a number of flavonoid-rich foods including tea[81], chocolate [85,86], apples[79,87],
313 onions[87], bran[79], red wine[79], grapefruit[79] and strawberries[79]. Tea and chocolate/cocoa intake
314 have been the focus of attention with a number of meta-analyses performed to date. The
315 relationship between tea consumption and coronary heart disease was examined by Peters et
316 al[88] in a meta-analysis of 10 cohort studies and 7 case-control studies. Their findings indicated
317 an overall reduction in risk (11%) with consumption of 3 cups of green or black tea per day.
318 However a recent meta-analysis of 18 studies showed no evidence of a protective effect of
319 black tea, while a small reduced risk was observed for green tea consumption[89]. A meta-
320 analysis examining the relationship between tea consumption and risk of stroke observed that
321 individuals consuming more than 3 cups of tea per day had a 21% lower risk of stroke

compared to those drinking less than 1 cup per day[90]. A meta-analysis of chocolate consumption and risk of cardiovascular disease observed that high chocolate consumption was associated with a 37% reduction in risk of cardiovascular disease and a 29% reduction in risk of stroke compared to low chocolate consumption[91].

Overall, epidemiological studies examining the association of flavonoid intake with cardiovascular disease suggest a protective effect. Apart from the fact that no cause and effect relationship can be established from epidemiological studies, these studies have a number of other limitations. The first limitation relates to the food frequency questionnaire (FFQ). The FFQ's used may not have been designed to measure flavonoid intake and may, therefore, be inaccurate in measurements of dietary flavonoid exposure. This could be due to missing foods from the FFQ that have flavonoids, flavonoids hidden in sauces and soups as well as foods that are grouped together that differ in their flavonoid profile. Other problems with FFQs include recall bias as well as error of measurement in an exposure of interest. An over or underestimation of flavonoid exposure could result from variances in flavonoid concentration in food due to growing, storage and cooking methods. Two new databases of polyphenol composition of foods have been developed, the Phenol-Explorer[92] and the United States Department of Agriculture (USDA) databases. There are, however, inherent differences between these databases, including that the two databases use different source data, with different origins of foods, for each individual flavonoid, such that they will give different estimates of intake.

The second limitation concerns the possibility of residual confounding in that intake of flavonoids may be positively associated with a healthy lifestyle. People with a higher flavonoid intake are more likely to be non-smokers, have lower intakes of total and saturated fats and have a lower BMI[80,93,94]. The third limitation relates to the lack of a reliable biomarker for polyphenol intake although several have been highlighted (reviewed by

Zamora-Ros *et al*.[95]). The final limitation concerns meta-analyses of epidemiological studies and the potential for publication bias. Studies showing an effect are more likely to be published and therefore included in a meta-analysis. Regardless of these limitations, there is evidence of a cardiovascular protective effect with high flavonoid intake that needs to be investigated further.

Blood pressure

High blood pressure is a major risk factor for cardiovascular disease. A number of lifestyle measures are recommended to lower blood pressure such as exercise, smoking cessation, reducing salt intake, lowering alcohol consumption and increasing fruit and vegetable intake[96]. The blood pressure lowering effects of a diet high in fruit and vegetables has been attributed, in part, to its high flavonoid content. Wide scale research examining the effects of different flavonoid-rich foods on blood pressure, however, have had mixed results. Meta-analyses have been performed for tea, cocoa and chocolate, soy products and grape seed extract **(Table S1** available in Supporting Information online). Increased blood pressure was observed with acute black tea intake and no effect on blood pressure was observed with both chronic black and green tea intake[59]. Decreased blood pressure was seen with chronic cocoa and chocolate intake[97,98] and no effect on blood pressure was seen with acute intake[97]. A decrease in blood pressure was observed after chronic soy intake in hypertensive individuals and no effect was observed in normotensives[99]. A decrease in diastolic blood pressure was observed with more than 2 week intake of grape seed extract[100]. Other flavonoid-rich food examined for effects on blood pressure include fruit juices such as pomegranate juice[101,102], red wine[103], grapes[104,105] and berries[106]. Differences in effects of the flavonoid-rich foods on blood pressure could reflect differences in bioavailability and bioactivity of the flavonoid subclasses as well as any inhibition, additive effects or synergism among the phytochemicals present. Of concern, however, is the considerable heterogeneity in results obtained from

randomised controlled trials examining the effects of a single flavonoid-rich food on blood pressure. One possible reason is the different doses of specific flavonoids present in the interventions. Indeed, a recent meta-analysis examining dose of (-)-epicatechin in ingested cocoa products and effects on blood pressure found that between study differences could be accounted for using a nonlinear regression model that considered epicatechin dose[107].

Randomised controlled trials examining effects of pure flavonoids on blood pressure could address these limitations but there are few and mainly focus on quercetin with one study examining epigallocatechin-3-gallate (**Table S2** available in Supporting Information online). The majority of these studies focus on individuals at risk for cardiovascular disease including prehypertensives, stage 1 hypertensives and overweight individuals[108-111]. Overall, decreases in blood pressure are observed, however, the dose of flavonoids administered in some of these pure flavonoid studies exceed amounts that can be obtained from the diet. It should be noted that while the blood pressure reductions observed in randomised controlled trials with some flavonoid-rich foods are small, the impact on cardiovascular events in the population would be profound with a 2-3% reduction in risk expected for each mmHg reduction in blood pressure[112].

Endothelial function

Endothelial dysfunction can occur long before any vascular disease is detected and has a prognostic value independent of other cardiovascular risk factors. Interventions that reduce risk of cardiovascular disease such as smoking cessation, exercise and certain medications can reverse endothelial dysfunction[5]. A recent meta-analysis has shown that FMD is a predictor for future cardiovascular events[113]. Nutritional interventions that enhance endothelial function will, therefore, improve cardiovascular health and its outcomes. In this respect, intervention studies with flavonoid-rich foods have shown improvements in endothelial function as measured by FMD. Meta-analyses have been performed for tea[114], cocoa[97] and chocolate, and

soy products[115] (**Table S4 -** available in Supporting Information online). Improvements in FMD are consistent for tea and cocoa/chocolate interventions. While the meta-analysis of soy products showed an overall positive effect, beneficial effects are not consistently observed in randomised controlled trials. Other flavonoid-rich foods that have shown positive effects on endothelial function include apples[116], grape juice[117,118], dealcoholised red wine[119], blackcurrants[120] and red orange juice[121]. To date, only a single randomised controlled trial has examined the effects of a pure flavonoid, (-)-epicatechin, on endothelial function. Schroeter et al[122]observed a significant increase in FMD two hours after ingestion of both 1mg/kg and 2 mg/kg bw (-)-epicatechin in a limited number of subjects (n=6). (-)-Epicatechin could thus, in part, mediate the beneficial effects on endothelial function observed after consumption of cocoa/chocolate. (-)-Epicatechin is also present in red wine, red grapes, tea and apples.

Platelet function

Modulation of platelet activity is required to prevent platelet aggregation, a critical event in the development of vascular thrombosis with outcomes such as myocardial infarction and unstable angina. Cardiovascular disease can be prevented by antiplatelet therapy indicating that nutritional interventions that modulate platelet activity could improve cardiovascular health. A number of studies have examined the effects of flavonoid-rich foods on platelet aggregation, with mixed results[123]. These include cocoa/chocolate[124], tea[125-128], berry products[129-132], pomegranate juice[101] and sea buckthorn[133]. No meta-analysis or pure flavonoid *in vivo* studies have been performed to date. Pure flavonoid *ex vivo* studies have been performed however the clinical relevance of *ex vivo* platelet studies is not clear[134].

Nitric oxide as a key regulator of beneficial effects on vascular health

The exact mechanisms by which flavonoids exert beneficial effects on vascular health have yet to be elucidated. A number of potential pathways, however, have been highlighted by recent studies. These can be classified into general/nonspecific and specific mechanisms[135].

Both specific and nonspecific mechanisms can be further subdivided into NO- related and other effects (summarised in **Table 2**[135-150]). The general/nonspecific mechanisms are dependent on the antioxidant nature of phenolic groups while for specific mechanisms, particular flavonoid structural features are required[142]. It is now recognised that because flavonoids are poorly absorbed and extensively metabolised, their contribution to the total level of antioxidants in the body is negligible. Therefore a direct antioxidant is effect is unlikely[151]. It is also important to note that some of the possible mechanisms of action of flavonoids have been determined in animal and human cell lines using flavonoid aglycones or glycosides at micro- or millimolar concentrations. Flavonoids, however, generally appear in the circulation as metabolites in nanomolar concentrations. This highlights the importance of randomised controlled trials utilising flavonoid rich foods or pure flavonoids in determining mechanisms by which flavonoids improve vascular health.

Previous work by our group has demonstrated that quercetin and two of its major metabolites, methyl-quercetin and quercetin glucuronide can have significant beneficial effects *in vitro* and *ex vivo*. In particular, querectin and its metabolites were able to improve acetylcholine induced relaxation of isolated aortic rings, as well as protect against hyperchlorous acid-induced endothelial dysfunction. Further *in vitro* analysis using isolated human aortic endothelial cells, indicated that these effects were mediated via an AMPK pathway, a critical cellular energy sensor, and facilitated eNOS activity via enhance phosphorylation of the eNOS enzyme and subsequent production of NO[152].

In addition, utilising the ApoE knockout out mouse, an established animal model of atherosclerosis, we have also shown that quercetin can protect against oxidant-induce endothelial dysfunction, as well as attenuate the development of atherosclerosis following a high fat diet. The effects were associated with improvements in NO bioavailability, supporting our previous *in vitro* work. Furthermore, these protective effects appear to be

447 critically related to the arterial induction of heme oxygnase-1 (HO-1), an inducible enzyme
448 that can protect the vasculature from oxidative stress. Quercetin was shown to induce HO-1
449 expression in both human aortic endothelial cells and aorta removed from wild type mice.
450 Interestingly, quercetin was unable to protect against oxidant-induced endothelial dysfunction
451 in heterogenous HO-1 knockout mice, further supporting a role for HO-1 (unpublished data).

452 Clinical trials with flavonoid-rich foods have demonstrated an improvement in endothelium-
453 dependent vasodilatation measured by FMD, as described previously. Since FMD provides a
454 measure of in vivo endothelium-derived NO bioavailability, this signifies an improvement in
455 NO status. Indeed, in a study by Fisher et al[153], vasodilation after consumption of flavan-3-ol-
456 rich cocoa was reversed with a concomitant intravenous infusion of a specific nitric oxide
457 synthase inhibitor. Increases in measures of NO status have also been observed after both
458 acute and chronic intake of cocoa[146,154]. In a randomised clinical trial with healthy men and
459 women, improvements in FMD with a concomitant increase in NO status were observed after
460 intake of flavonoid-rich apples[116]. Additionally, a randomised cross-over trial with healthy
461 men, showed the acute intake of the pure flavonoids quercetin, (-)-epicatechin but not
462 epigallocatechin gallate resulted in increases in markers of NO status and a decrease in
463 endothelin-1[140]. These studies suggest that an important mechanism by which flavonoids
464 benefit vascular health is through effects on NO status. How exactly flavonoids exert both
465 acute and chronic effects on NO status is currently unresolved.

466 The acute, short-term, versus the chronic, longer-term, mechanistic effects of flavonoid intake
467 also need to be distinguished. Acute effects of flavonoid intake are reversible and correspond
468 with peak plasma levels of flavonoid metabolites. Acute effects of flavonoid intake include
469 decreases in blood pressure and improvements in FMD. The instant physiological responses
470 signify activation or inhibition of enzymes or other proteins. With regards to effects on NO
471 status, this could involve inhibition of enzymes that breakdown NO[155], resulting in prolonged

NO bioavailability, and/or activation of eNOS[156] with increased NO production. The relevance of the acute effects of flavonoid intake to risk of cardiovascular disease is not clear. Chronic, long term, effects of flavonoid intake such as decreases in blood pressure and improvements in FMD are observed without ingestion of the flavonoid 2 hours prior to measurement (removing the acute effect)[157]. Chronic effects could include activation or inhibition of enzymes or other proteins, as well as involve changes in gene expression and protein synthesis. With regards to effects on NO status, this could involve increased transcription of eNOS[156] as well as enzymes improving arginine availability (a NO precursor)[158]. Long-term vascular changes could result with a subsequent improvement in cardiovascular outcomes.

Toxicity

The well-publicised, wide ranging health effects of flavonoids has initiated the development of dietary supplements with megadoses of flavonoids. Quercetin, for example, as a dietary supplement can be taken in a dose of 1000 mg/day. This is 20 times higher than the amount obtained in a typical vegetarian diet[159]. Isoflavone supplements with varying doses are also popular[160]. Of concern, the potential risk of consuming flavonoids, particularly in high doses, is not well understood and the "safety of elevated intakes cannot be assumed"[161]. A number of adverse effects, primarily in *in vitro* and animal studies, have been observed for different flavonoids and include anti-nutritional effects, thyroid toxicity, drug interactions, carcinogenicity and developmental effects (summarised in **Table 3**[58,160,161]). An assessment of toxicity is complicated. There are a large number of different naturally occurring flavonoids as well as a deficiency in accurate dietary intake data. Additionally, observational epidemiological studies with good dietary intake records generally look at health effects not adverse events. Studies assessing hazard, risk and safety of flavonoid consumption are lacking[58]. Many commonly consumed foods are rich in flavonoids, thus maintaining

497 flavonoid intakes at levels consistent with that of a typical vegetarian diet is generally
498 considered safe[58,160].

499 **NITRATE AND VASCULAR FUNCTION**

500 **Dietary sources**

501 Vegetables and drinking water are the primary sources of nitrate in the diet. Nitrate is
502 absorbed effectively with a bioavailability of 100%[162]. Intake of nitrate, therefore, is
503 dependent on nitrate concentration in vegetables, the amount of vegetables consumed as well
504 as nitrate content of drinking water[163]. The nitrate content in vegetables varies greatly and is
505 highly dependent on genetic, environmental and agricultural factors[164-167]. Nitrate-rich
506 vegetables include beetroot, lettuce, rocket and spinach (>250 mg/100g). Other vegetables,
507 such as peas, potato and tomato, contain lower amounts of nitrate (<20 mg/100g) but due to
508 the quantity consumed contribute significantly to total nitrate intake[168]. The nitrate content of
509 water varies considerably[169] and is highly regulated by most countries because of health
510 concerns.

511 Due to a large variation in the nitrate content of vegetables, total nitrate consumption is
512 difficult to determine. Estimates of mean daily intake range from 0.4 to 2.6 mg/kg (31 to 185
513 for a 70 kg adult)[170]. Individual daily nitrate intakes range from less than 20 mg to greater
514 than 400 mg[166,167]. The Acceptable Daily Intake (ADI) for nitrate set by the European Food
515 Safety Authority is 3.7 mg/kg (260 mg for a 70 kg adult) due to concerns of toxicity in
516 relation to methaemoglobinaemia, colorectal cancer and cardiovascular disease. Individuals
517 who follow the Dietary Approaches to Stop Hypertension (DASH) diet, however, could
518 consume as much as 1000 mg/d. It seems unlikely that a diet rich in high-nitrate vegetables
519 would have detrimental health effects.

520 **Vascular benefits of nitrate intake**

Epidemiological studies suggest an inverse association between consumption of green leafy vegetable, high in dietary nitrate, and the risk of cardiovascular disease. Cardiovascular protection by dietary nitrate could occur by enhancing NO status through the enterosalivary nitrate-nitrite-NO pathway with effects on vascular health. Indeed clinical studies have demonstrated lowering of blood pressure, improvement in endothelial function and decreased arterial stiffness with nitrate intake. There is still concern, however, about possible detrimental health effects with nitrate intake.

Epidemiological evidence

There is a close association between the Mediterranean and the traditional Japanese diets with a reduced incidence of cardiovascular disease[171-173]. This could be partially due to their high nitrate content[174,175]. Indeed, these diets may contain as much as 20 times the nitrate present in a typical Western Diet[176]. Additionally, green leafy vegetables, which are high in dietary nitrate, have been shown to be protective against coronary heart disease, stroke and type 2 diabetes[3,4,177]. While clinical trials support the hypothesis that nitrate intake is protective, no epidemiological study has examined nitrate intake specifically with cardiovascular health or disease. Possible reasons include the close correlation of nitrate with vegetable and therefore other nutrient consumption[178]; large within-foods variation of nitrate concentration as well as lack of a biomarker for nitrate intake.

Blood pressure

The evidence of decreased NO production in hypertension[10] together with the discovery of the enterosalivary nitrate-nitrite-NO pathway[179-181] raised the possibility that nitrate could partially account for the blood pressure lowering effect of green leafy and cruciferous vegetables[3,4]. To date, more than 25 studies have examined the effect on blood pressure of an acute or chronic dose of nitrate whether in the form of beetroot juice, high green leafy vegetable diet or the nitrate salts (**Table 4**[32,39,116,172,182-199]). While most studies show a

546 reduction in blood pressure, the effects are not consistent. Some studies observe a reduction
547 in either systolic or diastolic blood pressure while others observe a decrease in both. A
548 recent meta-analysis of 15 clinical trials found a significant association between nitrate
549 consumption and a decrease in systolic blood pressure[200]. Of the total 25 studies conducted,
550 only 3 have examined effects on participants at risk for cardiovascular disease. A study with
551 subjects with Peripheral Artery Disease observed a decrease in diastolic blood pressure 120
552 min after beetroot juice consumption[191]. A reduction in systolic blood pressure in elderly
553 volunteers with a moderate cardiovascular disease risk was observed post 28 day sodium
554 nitrate intake[201]. No effect on blood pressure was observed after 14 day beetroot juice intake
555 in volunteers with Type 2 diabetes[187]. Whether dietary nitrate lowers blood pressure in other
556 populations at risk for cardiovascular disease, such as those with hypertension, still needs to
557 be established.

558 Effects on blood pressure are observed with concomitant increases in plasma nitrate and
559 nitrite[32,39,184,189,195]. Additionally it has been demonstrated that interrupting the
560 enterosalivary nitrate-nitrite-NO pathway post a nitrate dose by use of antibacterial
561 mouthwash or spitting prevents the rise in plasma nitrite and associated reduction in blood
562 pressure[30,32]. Interestingly, a recent study in healthy volunteers has demonstrated that
563 antibacterial mouthwash use for 7 days resulted in a significant decrease in salivary and
564 plasma nitrite on a background (24 hour) low nitrate diet with increased systolic and
565 diastolic blood pressure[202]. This study confirms that nitrate produced endogenously as an
566 end product of NO metabolism is recycled back through the nitrate-nitrite-NO pathway
567 contributing to total systemic nitrite, the circulating NO pool and physiological regulation
568 of blood flow.

569 Results of a recent experiment in rats demonstrate the possible existence of cross-talk
570 between the L-arginine NOS pathway and the nitrate-nitrite-NO pathway[203]. Long term high

571 dose nitrate supplementation was associated with increased blood pressure, a down-
572 regulation of eNOS and decreased cGMP. Whether this occurs in humans still needs to be
573 determined.

574 ***Endothelial function***

575 Through the enterosalivary nitrate-nitrite-NO pathway, dietary nitrate is an alternate source
576 of vasodilatory NO. Thus dietary nitrate could improve endothelial function. Indeed 7 of 9
577 studies conducted to date have observed significant improvements in FMD post nitrate intake
578 in the form of beetroot juice, spinach or the nitrate salts (**Table 5**[32,116,182,187,189,198,204,205]). Of
579 the 9 studies conducted, 3 examined effects in participants at risk for cardiovascular disease.
580 In healthy but overweight men the postprandial decrease in FMD after a meal was prevented
581 with concomitant intake of beetroot juice[205]. An improvement in FMD in elderly volunteers
582 with a moderate cardiovascular disease risk was observed post 28 day sodium nitrate
583 intake[201]. No effect on FMD was observed after 14 day beetroot juice intake in volunteers
584 with Type 2 diabetes[187].

585 In two of these studies, Webb et al [32]and Kapil et al[189] demonstrated that dietary nitrate
586 prevented ischaemia-induced endothelial dysfunction (measured by FMD) after 20 min
587 occlusion of blood flow. Ischemia reperfusion injury, the tissue damage on restoration of
588 blood flow after a period of ischaemia or lack of oxygen, could have severe consequences
589 and in the heart and brain is a major cause of death and morbidity[206]. When oxygen tension
590 and pH falls, rendering the L-arginine-NOS pathway inactive, nitrite reduction to NO is
591 enhanced. Thus increasing plasma and tissue nitrite levels could augment NO status and
592 prevent or reduce the damage caused by ischaemia-reperfusion injury. Indeed, this has been
593 demonstrated in a number of animal models with administration of nitrite[207-212].

594 ***Arterial Stiffness***

NO influences vascular tone and, therefore, arterial stiffness. To date only 3 studies have examined the effect of dietary nitrate on arterial stiffness. Bahra et al[182] observed a significant decrease in pulse wave velocity (PWV) in healthy volunteers (n=14) 3 hours post 500 mg nitrate (potassium nitrate) ingestion. We recently observed (Liu et al[197]) increased large artery elasticity, but no effect on PWV in healthy volunteers (n=28) after 220 mg nitrate (spinach). Rammos et al[201] observed a significant decrease in PWV in elderly volunteers with moderate cardiovascular risk (n=11) after 4 week consumption of 900 mg nitrate (sodium nitrate) daily. As all studies observed concomitant decreases in SBP, it is possible that this accounts for the decrease in arterial stiffness observed. While these studies provide promising evidence of an effect of dietary nitrate on arterial stiffness, more studies are required to confirm these findings.

Toxicity

Despite the increasing evidence of health benefits with nitrate intake, there is still concern among some researchers regarding potential detrimental health effects such as cancer, cardiovascular disease and methaemoglobinaemia.

The demonstration that dietary nitrate has the potential to form carcinogenic *N*-nitrosamines[33,213] together with a study reporting dietary nitrite caused lymphomas in rats[214] sparked extensive research examining a possible link between dietary nitrate consumption and cancer. A review of all these studies by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2003, International Agency for Research on Cancer in 2006, and the European Food Safety Authority (EFSA) in 2008, however, found no evidence that dietary nitrate intake increases cancer risk. Indeed, an increased mortality from cancer is not observed with a diet rich in fruit and vegetables containing levels of nitrate far exceeding those recommended by the WHO[177]. Vegetables contain high levels of compounds such as vitamins C and E which could prevent *N*-nitrosamine formation.

The concern that dietary nitrate may be related to cardiovascular diseases arises from evidence of an association between consumption of processed meats and cardiovascular disease. A recent meta-analysis has found an association between processed meat intake and incidence of cardiovascular disease[215]. Whether this is related to the presence of nitrate and nitrite, added as an antimicrobial agent, flavour enhancer and colourant, and their potential to form nitrosamines is unknown. There is little evidence to support this association.

The possible relationship between methaemoglobinaemia (blue baby syndrome) and nitrate intake was first raised in the 1940s when methaemoglobinaemia was observed in infants fed formula made with well water that had a high nitrate concentration[216]. It has been argued since that nitrate was simply a marker of faecal contamination and not the actual cause[217]. Indeed, methaemoglobinaemia was not observed in infants and adults given a high dose of nitrate (50 or 100 mg nitrate/kg/day)[218]. Western Countries still spend millions of dollars annually to lower the nitrate content of drinking water to levels established in 1970 and reviewed in 2004 by the World Health Organisation. Whether these recommended levels of nitrate in drinking water should be raised is a controversial issue[219].

DIETARY FLAVONOIDS AND NITRATE: POTENTIAL FOR ADDITIVE BENEFITS

Evidence is convincing that changes in dietary behaviour, such as increasing fruit and vegetable intake, will reduce the incidence of cardiovascular disease. The multitude of bioactive phytochemicals present in fruit and vegetables has been the focus of intense research to identify the “magic bullet” or at least the main contributing compounds. While it is important to study the effects of individual phytochemicals, it is uncertain, however, whether a single phytochemical will have the same benefit as when part of a whole food or combinations of foods are consumed. Indeed, combinations of phytochemicals may exhibit

644 additive, synergistic or antagonistic interactions. Additive and synergistic interactions could
645 occur when phytochemicals act on complementary but different molecular pathways.

646 The possibility that simultaneous ingestion of dietary nitrate and flavonoids could have an
647 additive or even synergistic effect on vascular health comes from the observation that they
648 both enhance NO production via different mechanisms as well as from studies demonstrating
649 that flavonoids enhance the reduction of nitrite to NO. Dietary nitrate contributes to the
650 circulating pool of nitrite and NO through the nitrate-nitrite-NO pathway. While the exact
651 mechanisms of protective action by flavonoids has yet to be confirmed, evidence suggests that
652 flavonoids modulate NO metabolism through the L-arginine NOS pathway. *In vitro* studies
653 and *in vivo* experiments suggest that flavonoids could also mediate the direct bioconversion of
654 nitrite to NO. These studies have demonstrated that flavonoids, in the acidic conditions of the
655 stomach, can enhance the production of NO from salivary nitrite[145,148-150] which can diffuse
656 across the stomach wall and induce local muscle relaxation[220,221]. Since salivary nitrite is
657 increased after nitrate consumption, polyphenols could, theoretically, enhance NO production
658 after a nitrate rich meal. Whether this occurs in the circulation is unknown. In a recent study
659 we examined the combined effect of flavonoid-rich apples and nitrate-rich spinach on NO
660 status, blood pressure and FMD in 30 healthy men and women[116]. While significant effects on
661 NO status, SBP and FMD were observed when the flavonoid-rich apples and nitrate-rich
662 spinach were given independently, no synergistic or even additive effect was observed with
663 the combination. The reduced, but still significantly increase in NO status, SBP and FMD
664 could be due to increased production of NO from nitrite in the stomach with less nitrite then
665 available for absorption into the circulation. This possibility requires further investigation.

666 **CONCLUSION**

667 Undoubtedly NO plays a pivotal role in cardiovascular health. Cardiovascular disease is
668 associated with endothelial dysfunction and a decreased production and /or bioavailability of

NO. An augmentation of NO status by components of a fruit and vegetable diet, such as the flavonoids and nitrate, could have a significant impact on risk of cardiovascular disease with increased consumption. In this regard, evidence from studies examining the effect of flavonoids and nitrate on cardiovascular risk factors is promising. The molecular mechanisms by which flavonoids are cardioprotective are not completely understood. Recent evidence suggests both non-specific and specific effects through NO pathways. Flavonoids are, however, highly metabolised so establishing the absorption kinetics, metabolism and identity of flavonoid metabolites is imperative to understanding their cardioprotective effects. Future studies are still required to determine if flavonoids are a potential new therapy for those at risk for cardiovascular disease. In particular, randomised clinical trials with pure flavonoids are needed to determine which flavonoids (and their respective doses) are responsible for the cardioprotective benefits of a fruit and vegetable diet. The observed benefits of dietary nitrate on cardiovascular health are not without controversy due to a lingering concern over possible detrimental health effects. Evidence from clinical trials, however, suggests that increased nitrate intake is an effective strategy in cardiovascular disease prevention. Whether dietary nitrate would be an effective treatment for people with hypertension or impaired vascular function still needs to be determined. Also worth further investigation is the relationship between flavonoids and nitrate as whole foods and not isolated compounds are consumed.

ACKNOWLEDGEMENTS

Funding. There was no external funding for this review. CP Bondonno acknowledges the support of an Australian Postgraduate Award. NC Ward acknowledges the support of a MRF/UWA Fellowship. JM Hodgson was supported by an NHMRC Senior Research Fellowship.

Declaration of interest. The authors have no relevant interests to declare.

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Figure legend

Figure 1: **Classification of phytochemicals.** There are 5 main classes of phytochemicals. The polyphenols can be further divided into **flavonoids** and **non-flavonoids**. Some example food sources are illustrated.

Figure 2: **Absorption and metabolism of flavonoids.** Quercetin glycosides represent the absorption and metabolism of flavonoid glycosides. Epicatechin represents the absorption and metabolism of the flavonoid subclass, the flavan-3-ols.

Figure 1

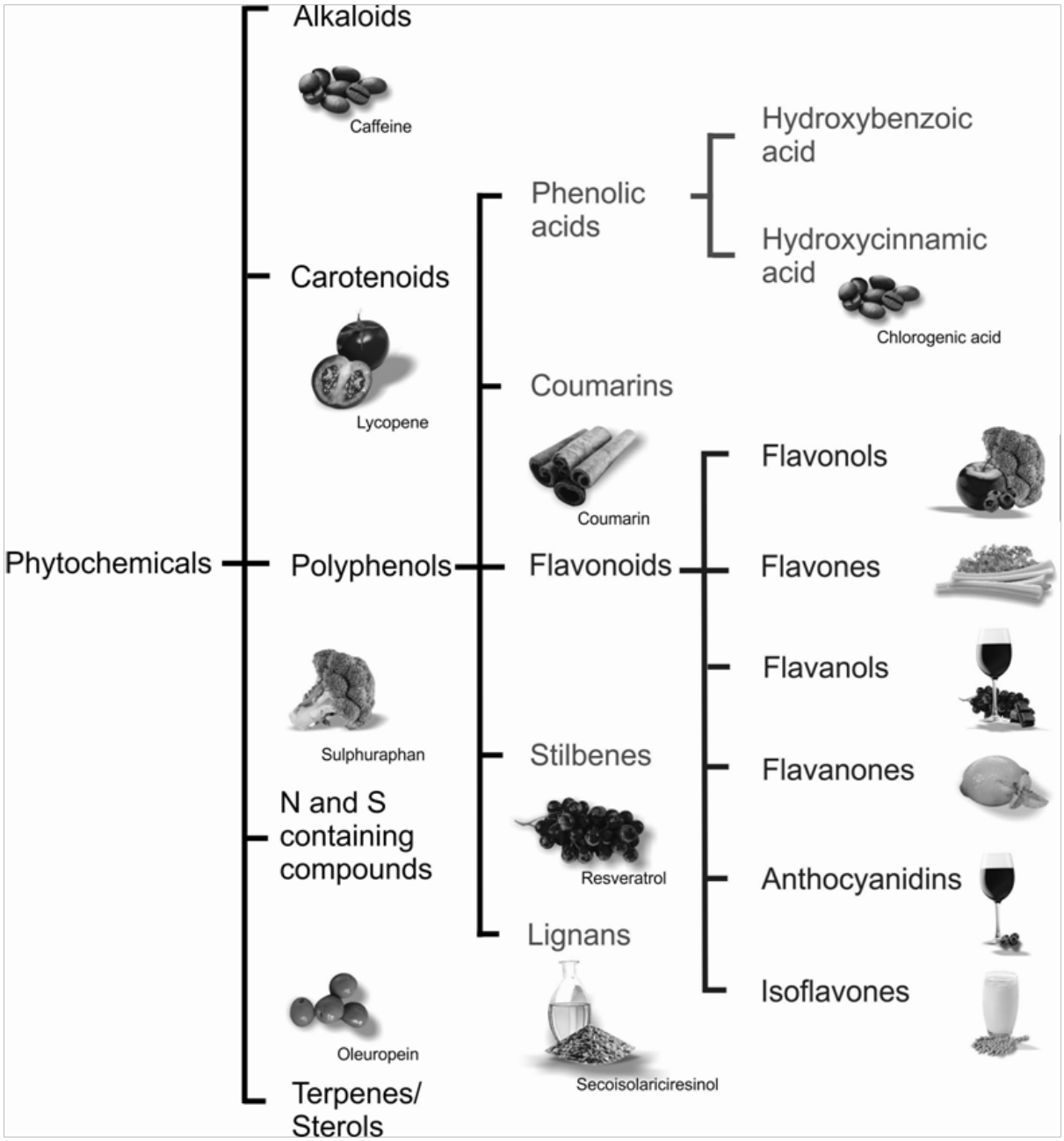

Phytochemicals
Alkaloids
Caffeine
Carotenoids
Lycopene
Polyphenols
Sulphuraphan
N and S containing compounds
Oleuropein
Terpenes/ Sterols
Phenolic acids
Hydroxybenzoic acid
Hydroxycinnamic acid
Chlorogenic acid
Coumarins
Coumarin
Flavonoids
Stilbenes
Resveratrol
Lignans
Secoisolariciresinol
Flavonols
Flavones
Flavanols
Flavanones
Anthocyanidins
Isoflavones

Figure 2.

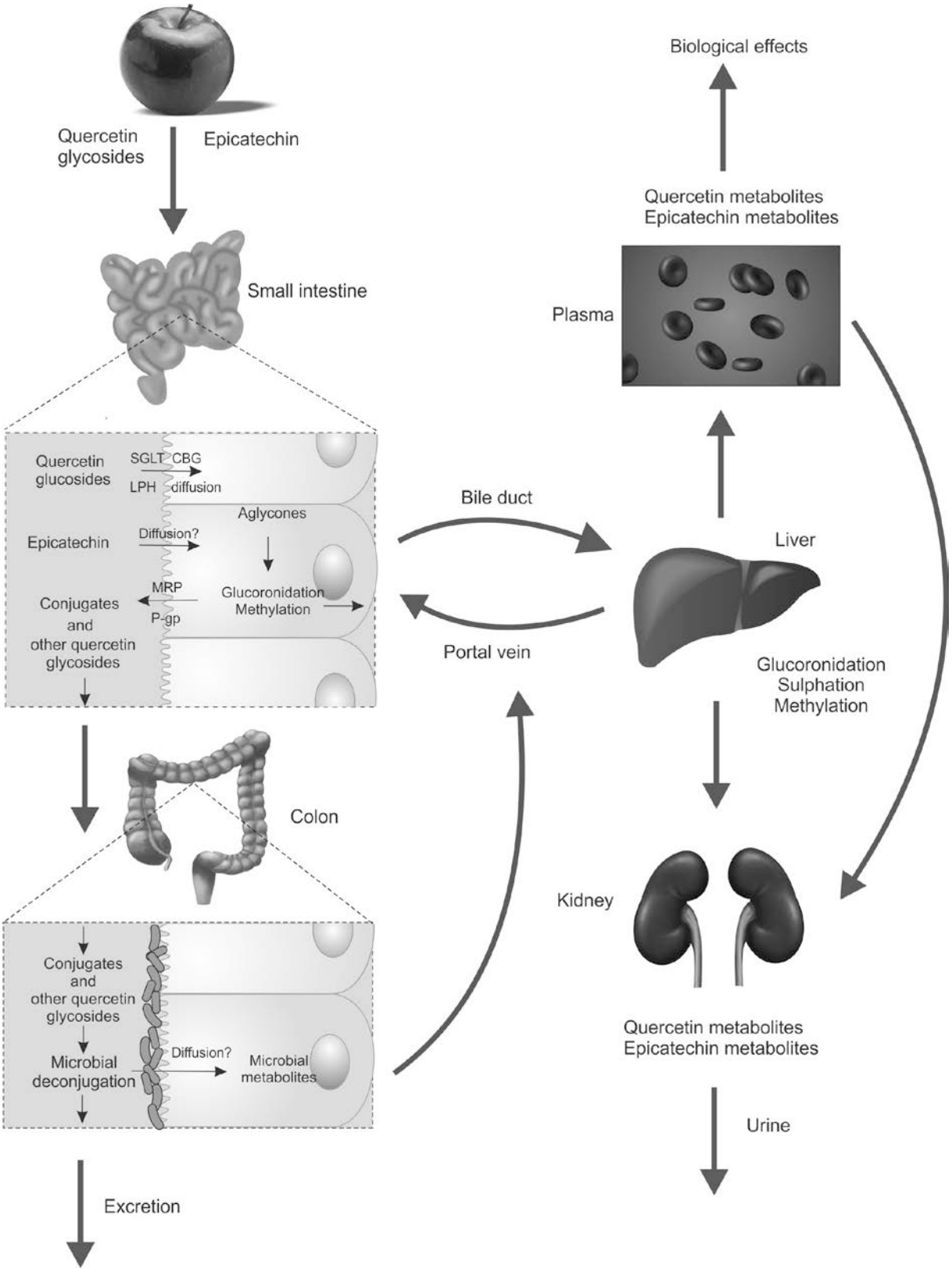

Biological effects
Quercetin metabolites
Epicatechin metabolites
Plasma
Quercetin glycosides
Epicatechin
Small intestine
Quercetin glucosides
SGLT CBG
LPH diffusion
Aglycones
Epicatechin
Diffusion?
MRP
P-gp
Conjugates and other quercetin glycosides
Glucoronidation
Methylation
Bile duct
Liver
Portal vein
Glucoronidation
Sulphation
Methylation
Colon
Kidney
Conjugates and other quercetin glycosides
Microbial deconjugation
Diffusion?
Microbial metabolites
Quercetin metabolites
Epicatechin metabolites
Urine
Excretion

Table 1: Flavonoid subclasses, prominent flavonoids and typical food sources[52,53]

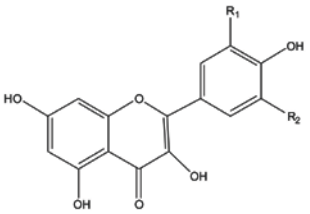
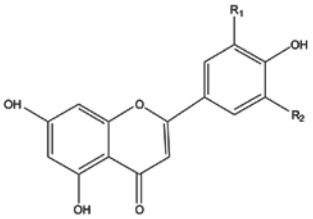
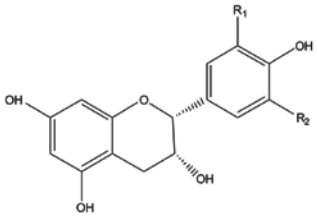
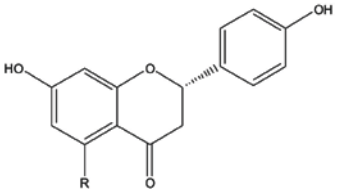
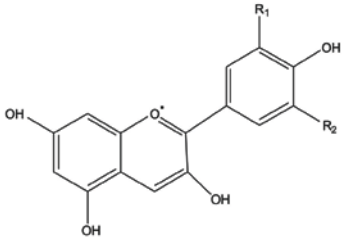
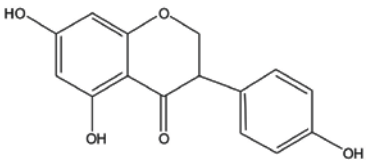
Subclass	Prominent flavonoids	Typical food source
Flavonols 	Isorhamnetin Kaempferol Quercetin Myricetin	Tea, apples, onions, curly kale, leeks, broccoli, blueberries, red wine,
Flavones 	Apigenin Luteolin	Parsley, celery
Flavan-3-ols 	(+)-Catechin (+)-Gallocatechin (-)-Epicatechin (-)-Epigallocatechin (-)-Epicatechin-3-gallate (-)-Epigallocatechin-3-gallate	Tea, red wine, red grapes, cocoa, chocolate, apricots
Flavanones 	Eriodictyol Hesperetin Naringenin	Citrus fruit, tomatoes, mint
Anthocyanidins 	Cyanidin Delphinidin Malvidin Pelargonidin Petunidin Peonidin	Berries, red wine
Isoflavones 	Daidzein Genistein Glycitein	Soybeans, soy foods, legumes

Table 2: Potential mechanisms whereby flavonoids exert effects on vascular health

Mechanism classification	Possible mechanism	Reference
General/nonspecific: NO related	Antioxidant effect: react with superoxide and other reactive oxygen species which could prevent NO and prostacyclin breakdown.	Gryglewski et al. (1987)[138]
	Antioxidant effect: protect tetrahydrobiopterin by scavenging peroxynitrite-derived free radicals, thus preventing eNOS uncoupling.	McCarty (2008)[141]
	Inhibition of xanthine oxidase, lipoxygenase and NADPH oxidase, enzymes which produce reactive oxygen species: prevent NO breakdown.	Mladenka et al. (2010)[142];Nijveldt et al. (2001)[144]
General/nonspecific: other	Antioxidant effect: inhibit lipid oxidation	Fraga et al. (2010)[135]
	Inhibition of xanthine oxidase, lipoxygenase and NADPH oxidase, enzymes which produce reactive oxygen species: other effects	Mladenka et al. (2010)[142];Nijveldt et al. (2001)[144]
	Chelation of metal ions: prevent formation of free radicals from metal catalysed reactions.	Morel et al. (1993)[143]
	Interaction with membrane lipids: could affect activity of membrane associated enzymes, ligand-receptor interactions, signal transduction and/or, ion/metabolite fluxes.	Fraga et al. (2010)[135]
Specific: NO related	Increased eNOS activity: enhanced NO production	Stoclet et al. (2004)[147]
	Increased eNOS expression: enhanced NO production	Stoclet et al. (2004)[147]
	Increased circulating nitrite: contributes to circulating NO pool and can be converted to NO when required	Schroeter et al. (2006)[137]; Balzer et al. (2008)[139]; Heiss et al. (2003)[146]
	Bioconversion of nitrite to NO: occurs in stomach. NO can diffuse through the stomach wall inducing smooth muscle relaxation. Whether this occurs in circulation is unknown.	Peri et al. (2005)[145]; Takahama et al. (2002)[148];

		Takahama et al (2010)[149]; Volk et al. (2009)[150]
	Inhibition of ACE activity: involved in regulation of renin-angiotensin system which could control NO production	Actis-Goretta et al. (2006)[136]
Specific: other	Enhanced prostacyclin production: vasodilation	Stoclet et al. (2004)[147]
	Enhanced endothelium-derived hyperpolarizing factor (EDHF) production: vasodilation	Stoclet et al. (2004)[147]
	Inhibition of endothelin-1 synthesis: vasodilation	Loke et al. (2008)[140]

Table 3: Observed adverse effects of flavonoids (summarised from [58,160,161]).

Effect	Observation
Antinutritional:	
Reduced glucose uptake	Observed in animal studies. Could have a protective effect by slowing absorption of glucose after a meal.
Impaired food utilisation	Proanthocyanidins interfere with protein utilisation in animal and human studies. Impaired lipid and carbohydrate metabolism observed with high flavonoid intakes in animal studies.
Impaired mineral absorption	Impairment of nonheme iron absorption observed in human studies. Heme iron absorption inhibited *in vitro* studies. Possible interaction between flavonoids and copper and manganese.
Impaired folate uptake	Observed *in vitro* studies. Little evidence of an effect in humans.
Vitamin C transport inhibition	Observed *in vitro* and animal studies.
Thyroid toxicity and goitrogenic activity	Observed *in vitro* and animal studies. No evidence of an effect in humans.
Drug interactions	*in vitro* and animal studies suggest: interactions with various cytochrome P450 monooxygenase (CYP) isoforms. interactions with phase II enzymes. interactions with drug transporters.
Genotoxicity/carcinogenicity	Observed *in vitro* and animal studies at high doses and concentrations.
Developmental effects	Possible association with infant acute myeloid leukemia (through effects on DNA topoisomerase II) but associated with a reduced risk for all leukemias.

Table 4: Summary of intervention studies examining both the acute and chronic effect of nitrate on blood pressure

References	Nitrate Source	Acute/Chronic	Nitrate Dose	Subject characteristics and number	BP Effect
Webb et al. (2008)[32]	Beetroot juice	Acute	1400 mg	Healthy M & F (n=14)	SBP, DBP, MAP ↓
Lansley et al. (2011)[193]		Acute	385 mg	Healthy M (n=9)	SBP ↓
Kapil et al. (2010)[189]		Acute	350 mg	Healthy M & F (n=14)	SBP ↓
Vanhatalo et al. (2010)[199]		Acute	322 mg	Healthy M & F (n=8)	SBP, DBP, MAP ↓
Kenjale et al. (2011)[191]		Acute	560 mg	PAD M & F (n=8)	DBP ↓
Coles and Clifton (2012)[186]		Acute	465 mg	Healthy M & F (n=30)	SBP ↓ M only
Kukadia et al. (2013)[192]		Acute	400 mg	Healthy M & F (n=9)	Central SBP ↓
Bailey et al. (2009)[184]		Chronic (6 d)	340 mg	Healthy M (n=8)	SBP ↓
Bailey et al. (2010)[183]		Chronic (6 d)	316 mg	Healthy M (n=7)	SBP, DBP, MAP ↓
Lansley et al. (2011)[194]		Chronic (6 d)	385 mg	Healthy M (n=9)	SBP ↓
Vanhatalo et al. (2010)[199]		Chronic (15 d)	322 mg	Healthy M & F (n=8)	SBP, DBP, MAP ↓
Gilchrest et al. (2013)[187]		Chronic (14 d)	465 mg	T2DM M & F (n=27)	No effect
Kelley et al. (2013)[190]		Chronic (3 d)	595 mg	Healthy M & F (n=12)	SBP, DBP ↓
Cermak et al. (2012)[185]		Chronic (6 d)	500 mg	Healthy M (n=12)	No effect
Hobbs et al. (2012)[188]	Beetroot juice (dose response)	Acute	143 mg	Healthy M (n=4)	SBP, DBP ↓
			353 mg	Healthy M (n=4)	SBP, DBP ↓
			707 mg	Healthy M (n=4)	SBP, DBP ↓
Hobbs et al. (2012)[188]	Red beetroot enriched bread	Acute	112 mg	Healthy M (n=5)	SBP ↓
	White beetroot enriched bread		99 mg	Healthy M (n=5)	No effect

Bondonno et al. (2012)[116]	Spinach	Acute	182 mg	Healthy M & F (n=30)	SBP ↓
Liu et al. (2013)[197]			220 mg	Healthy M & F (n=26)	SBP ↓
Sobko et al. (2010)[172]	Japanese traditional diet	Chronic (10 d)	18.8 mg/kg (±1200 mg)	Healthy M & F (n=25)	DBP ↓
Larsen et al. (2006)[39]	Sodium nitrate	Chronic (3 d)	6.2 mg/kg (±400 mg)	Healthy M & F (n=17)	DBP, MAP ↓
Larsen et al. (2007)[195]		Chronic (3 d)	6.2 mg/kg (±400 mg)	Healthy M (n=9)	SBP, DBP ↓
Rammos et al. (2013)[201]		Chronic (28 d)	12.75 mg/kg (±900 mg)	Elderly with moderate CV risk (n=11)	SBP ↓
Kapil et al. (2010)[189]	Potassium nitrate	Acute	1488 mg	Healthy M & F (n=21)	SBP, DBP ↓
Lidder at al. (2011)[196]		Acute	1488 mg	Healthy M & F (n=8)	No effect
Bahra et al. (2012)[182]		Acute	500 mg	Healthy M & F (n=14)	SBP ↓

Table 5: Summary of intervention studies examining both the acute and chronic effect of nitrate on measures of FMD

References	Nitrate Source	Acute/Chronic	Nitrate Dose	Subject characteristics and number	FMD Effect
Webb et al. (2008)[32]	Beetroot juice	Acute	1400 mg	Healthy M & F (n=14)	FMD↑
Kapil et al. (2010)[189]	Potassium nitrate	Acute	1488 mg	Healthy M & F (n=12)	FMD↑
	Beetroot juice		350 mg	Healthy M & F (n=12)	FMD↑
Bondonno et al. (2012)[116]	Spinach	Acute	182 mg	Healthy M & F (n=30)	FMD↑
Heiss et al. (2012)[204]	Sodium nitrate	Acute	1000 mg	Healthy M & F (n=10)	FMD↑
Bahra et al. (2012)[182]	Potassium nitrate	Acute	500 mg	Healthy M & F (n=14)	FMD no effect
Gilchrest et al. (2013)[187]	Beetroot juice	Chronic (14 d)	465 mg	T2DM M & F (n=27)	FMD no effect
Rammos et al. (2013)[201]	Sodium nitrate	Chronic (28 d)	12.75 mg/kg (±900 mg)	Elderly with moderate CV risk (n=11)	FMD↑
Joris et al. (2013)[205]	Beetroot juice	Acute	500 mg	Healthy but overweight BMI 28-35 kg/m^2 (n=20)	FMD↑