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SPECIAL ARTICLE

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

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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; BH₄, 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. eNOS catalyses the synthesis of NO by the 5-electron oxidation of the terminal guanidine nitrogen atom of L-arginine with nicotinamide adenine dinucleotide 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.

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.

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). NO also reacts rapidly with superoxide to form peroxynitrite, a reaction thought to be responsible for the pathological effects associated with NO. 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.

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. Rassaf et al. have
demonstrated that when NO is administered intravenously, the systemic effects observed are mediated by the conversion of NO to RSNOs\textsuperscript{29}.

**Nitrate-nitrite-NO pathway**

An alternate source of NO is the recently described nitrate-nitrite-NO pathway\textsuperscript{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\textsuperscript{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\textsuperscript{34}. In the acidic environment of the stomach some salivary nitrite is reduced to NO with localised gastrointestinal benefits\textsuperscript{35-37}. The remaining nitrite is absorbed into the bloodstream\textsuperscript{38} and, with nitrite formed as a by-product of NO metabolism, becomes a source of NO\textsuperscript{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\textsuperscript{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 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. Indeed, increased local arterial stiffness, elevated blood pressure as well as reduced blood flow are observed after inhibition of NO synthesis.

NO maintains vascular integrity by suppressing platelet aggregation, leukocyte migration, cellular adhesion to the endothelium as well as vascular smooth muscle cell proliferation. 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. 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.

**FLAVONOIDS AND VASCULAR FUNCTION**

Findings from epidemiological studies, 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, 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 glucuronides^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/variet, agricultural methods, growth environment, time of harvest, method of harvesting, storage
conditions as well as post-harvest processing and cooking methods\textsuperscript{52,58}. The normal dietary intake of particular cultures determines which foods and consequently which flavonoid subclasses are consumed. The primary dietary supply of flavonoids in western society includes tea, red wine, chocolate, cocoa, fruit, vegetables and legumes while in Japan and Indonesia, soy and soy foods are highly consumed, which provides high levels of isoflavones in the diet. There is also evidence to suggest that intake of certain subclasses and specific flavonoids may be more important than total flavonoid intake with regard to potential health benefits. Additionally, there is a wide variation in the bioavailability of flavonoid subclasses with some reaching higher biologically active concentrations than others\textsuperscript{53,58}.

**Absorption, metabolism and bioavailability**

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

The majority of flavonoids occur naturally as glycosides apart from the flavan-3-ols, which exist as aglycones. The absorption kinetics of the flavonoid glycosides, therefore, is quite different to that of flavan-3-ols. Absorption of flavonoid glycosides first requires the release of the aglycone by hydrolysis. This process typically occurs in the small intestine\textsuperscript{60} or later in the colon. In the small intestine, hydrolysis occurs by action of enteric membrane bound lactase-phlorizin-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”)\textsuperscript{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”)\textsuperscript{62}. Results of recent studies, however, seem to indicate that the “transport/CBG” pathway of flavonoid absorption may not exist\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{68}. Oligomers with a DP greater than 2\textsuperscript{68} and larger molecular weight proanthocyanidins are not absorbed and reach the colon where they are degraded by microflora to phenolic acids\textsuperscript{69}. A small proportion of these phenolic acids can be absorbed and may have bioactivity \textit{in vivo}.

It is well established that flavonoids undergo extensive metabolism after ingestion\textsuperscript{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. 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. Once in the blood stream, the conjugates rapidly reach the liver where they are further methylated, glucuronidated, or sulphated as part of phase II liver metabolism. 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 (entero-hepatic circulation) will be degraded in the colon by colonic microflora. 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. 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, 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. A large amount of unidentified metabolites may be present in the plasma.

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.

**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. Since this publication, 4 additional prospective studies have examined total flavonoid intake and cardiovascular disease mortality. Only five out of the 15 studies found a significant reduction in cardiovascular disease risk after multivariate adjustment with high flavonoid
intake compared to low flavonoid intake\textsuperscript{77,78}. However, when individual flavonoid subclasses are examined, a protective effect is observed for certain subclasses\textsuperscript{78}.

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

A large number of epidemiological studies have examined the relationship between flavonoid-rich foods and cardiovascular disease. Positive associations with reduced risk have been observed for a number of flavonoid-rich foods including tea\textsuperscript{81}, chocolate\textsuperscript{85,86}, apples\textsuperscript{79,87}, onions\textsuperscript{87}, bran\textsuperscript{79}, red wine\textsuperscript{79}, grapefruit\textsuperscript{79} and strawberries\textsuperscript{79}. Tea and chocolate/cocoa intake have been the focus of attention with a number of meta-analyses performed to date. The relationship between tea consumption and coronary heart disease was examined by Peters et al\textsuperscript{88} in a meta-analysis of 10 cohort studies and 7 case-control studies. Their findings indicated an overall reduction in risk (11%) with consumption of 3 cups of green or black tea per day. However a recent meta-analysis of 18 studies showed no evidence of a protective effect of black tea, while a small reduced risk was observed for green tea consumption\textsuperscript{89}. A meta-analysis examining the relationship between tea consumption and risk of stroke observed that 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.\textsuperscript{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\textsuperscript{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\textsuperscript{59}. Decreased blood pressure was seen with chronic cocoa and chocolate intake\textsuperscript{97,98} and no effect on blood pressure was seen with acute intake\textsuperscript{97}. A decrease in blood pressure was observed after chronic soy intake in hypertensive individuals and no effect was observed in normotensives\textsuperscript{99}. A decrease in diastolic blood pressure was observed with more than 2 week intake of grape seed extract\textsuperscript{100}. Other flavonoid-rich food examined for effects on blood pressure include fruit juices such as pomegranate juice\textsuperscript{101,102}, red wine\textsuperscript{103}, grapes\textsuperscript{104,105} and berries\textsuperscript{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. 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. 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.

**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. A recent meta-analysis has shown that FMD is a predictor for future cardiovascular events. 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, cocoa and chocolate, and...
soy products\textsuperscript{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\textsuperscript{116}, grape juice\textsuperscript{117,118}, dealcoholised red wine\textsuperscript{119}, blackcurrants\textsuperscript{120} and red orange juice\textsuperscript{121}. To date, only a single randomised controlled trial has examined the effects of a pure flavonoid, (-)-epicatechin, on endothelial function. Schroeter et al\textsuperscript{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\textsuperscript{123}. These include cocoa/chocolate\textsuperscript{124}, tea\textsuperscript{125-128}, berry products\textsuperscript{129-132}, pomegranate juice\textsuperscript{101} and sea buckthorn\textsuperscript{133}. No meta-analysis or pure flavonoid \textit{in vivo} studies have been performed to date. Pure flavonoid \textit{ex vivo} studies have been performed however the clinical relevance of \textit{ex vivo} platelet studies is not clear\textsuperscript{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\textsuperscript{135}.
Both specific and nonspecific mechanisms can be further subdivided into NO-related and other effects (summarised in Table 2). The general/nonspecific mechanisms are dependent on the antioxidant nature of phenolic groups while for specific mechanisms, particular flavonoid structural features are required. 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 effect is unlikely. 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, quercetin 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.

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

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

The acute, short-term, versus the chronic, longer-term, mechanistic effects of flavonoid intake also need to be distinguished. Acute effects of flavonoid intake are reversible and correspond with peak plasma levels of flavonoid metabolites. Acute effects of flavonoid intake include decreases in blood pressure and improvements in FMD. The instant physiological responses signify activation or inhibition of enzymes or other proteins. With regards to effects on NO status, this could involve inhibition of enzymes that breakdown NO\textsuperscript{155}, resulting in prolonged
NO bioavailability, and/or activation of eNOS\textsuperscript{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)\textsuperscript{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\textsuperscript{156} as well as enzymes improving arginine availability (a NO precursor)\textsuperscript{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\textsuperscript{159}. Isoflavone supplements with varying doses are also popular\textsuperscript{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”\textsuperscript{161}. A number of adverse effects, primarily in \textit{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\textsuperscript{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\textsuperscript{58}. Many commonly consumed foods are rich in flavonoids, thus maintaining...
flavonoid intakes at levels consistent with that of a typical vegetarian diet is generally considered safe\textsuperscript{58,160}.

**NITRATE AND VASCULAR FUNCTION**

**Dietary sources**

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

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

**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\textsuperscript{171-173}. This could be partially due to their high nitrate content\textsuperscript{174,175}. Indeed, these diets may contain as much as 20 times the nitrate present in a typical Western Diet\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{10} together with the discovery of the enterosalivary nitrate-nitrite-NO pathway\textsuperscript{179-181} raised the possibility that nitrate could partially account for the blood pressure lowering effect of green leafy and cruciferous vegetables\textsuperscript{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\textsuperscript{32,39,116,172,182-199}). While most studies show a
reduction in blood pressure, the effects are not consistent. Some studies observe a reduction in either systolic or diastolic blood pressure while others observe a decrease in both. A recent meta-analysis of 15 clinical trials found a significant association between nitrate consumption and a decrease in systolic blood pressure\textsuperscript{200}. Of the total 25 studies conducted, only 3 have examined effects on participants at risk for cardiovascular disease. A study with subjects with Peripheral Artery Disease observed a decrease in diastolic blood pressure 120 min after beetroot juice consumption\textsuperscript{191}. A reduction in systolic blood pressure in elderly volunteers with a moderate cardiovascular disease risk was observed post 28 day sodium nitrate intake\textsuperscript{201}. No effect on blood pressure was observed after 14 day beetroot juice intake in volunteers with Type 2 diabetes\textsuperscript{187}. Whether dietary nitrate lowers blood pressure in other populations at risk for cardiovascular disease, such as those with hypertension, still needs to be established.

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

Results of a recent experiment in rats demonstrate the possible existence of cross-talk between the L-arginine NOS pathway and the nitrate-nitrite-NO pathway\textsuperscript{203}. Long term high
dose nitrate supplementation was associated with increased blood pressure, a down-
regulation of eNOS and decreased cGMP. Whether this occurs in humans still needs to be
determined.

**Endothelial function**

Through the enterosalivary nitrate-nitrite-NO pathway, dietary nitrate is an alternate source
of vasodilatory NO. Thus dietary nitrate could improve endothelial function. Indeed 7 of 9
studies conducted to date have observed significant improvements in FMD post nitrate intake
in the form of beetroot juice, spinach or the nitrate salts ([Table S](#)) Of the 9 studies conducted, 3 examined effects in participants at risk for cardiovascular disease.

In healthy but overweight men the postprandial decrease in FMD after a meal was prevented
with concomitant intake of beetroot juice. An improvement in FMD in elderly volunteers
with a moderate cardiovascular disease risk was observed post 28 day sodium nitrate
intake. No effect on FMD was observed after 14 day beetroot juice intake in volunteers
with Type 2 diabetes.

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

**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\textsuperscript{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\textsuperscript{197}) increased large artery elasticity, but no effect on PWV in healthy volunteers (n=28) after 220 mg nitrate (spinach). Rammos et al\textsuperscript{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\textsuperscript{33,213} together with a study reporting dietary nitrite caused lymphomas in rats\textsuperscript{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\textsuperscript{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. 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 methaemoglobinemia (blue baby syndrome) and nitrate intake was first raised in the 1940s when methaemoglobinemia was observed in infants fed formula made with well water that had a high nitrate concentration. It has been argued since that nitrate was simply a marker of faecal contamination and not the actual cause. Indeed, methaemoglobinemia was not observed in infants and adults given a high dose of nitrate (50 or 100 mg nitrate/kg/day). 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.

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

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

CONCLUSION

Undoubtedly NO plays a pivotal role in cardiovascular health. Cardiovascular disease is 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

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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.
**Table 1:** Flavonoid subclasses, prominent flavonoids and typical food sources

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Prominent flavonoids</th>
<th>Typical food source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonols</td>
<td>Isorhamnetin</td>
<td>Tea, apples, onions, curly kale, leeks, broccoli, blueberries, red wine,</td>
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<tr>
<td></td>
<td>Kaempferol</td>
<td></td>
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<tr>
<td></td>
<td>Quercetin</td>
<td></td>
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<tr>
<td></td>
<td>Myricetin</td>
<td></td>
</tr>
<tr>
<td>Flavones</td>
<td>Apigenin</td>
<td>Parsley, celery</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td></td>
</tr>
<tr>
<td>Flavan-3-ols</td>
<td>(+)-Catechin</td>
<td>Tea, red wine, red grapes, cocoa, chocolate, apricots</td>
</tr>
<tr>
<td></td>
<td>(+)-Gallocatechin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-)-Epicatechin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-)-Epigallocatechin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-)-Epicatechin-3-gallate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-)-Epigallocatechin-3-gallate</td>
<td></td>
</tr>
<tr>
<td>Flavanones</td>
<td>Eriodictyol</td>
<td>Citrus fruit, tomatoes, mint</td>
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<tr>
<td></td>
<td>Hesperetin</td>
<td></td>
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<tr>
<td></td>
<td>Naringenin</td>
<td></td>
</tr>
<tr>
<td>Anthocyanidins</td>
<td>Cyanidin</td>
<td>Berries, red wine</td>
</tr>
<tr>
<td></td>
<td>Delphinidin</td>
<td></td>
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<tr>
<td></td>
<td>Malvidin</td>
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<tr>
<td></td>
<td>Pelargonidin</td>
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<td></td>
<td>Petunidin</td>
<td></td>
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<tr>
<td></td>
<td>Peonidin</td>
<td></td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Daidzein</td>
<td>Soybeans, soy foods, legumes</td>
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<tr>
<td></td>
<td>Genistein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycitein</td>
<td></td>
</tr>
<tr>
<td>Mechanism classification</td>
<td>Possible mechanism</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>General/nonspecific: NO related</td>
<td>Antioxidant effect: react with superoxide and other reactive oxygen species which could prevent NO and prostacyclin breakdown. Antioxidant effect: protect tetrahydrobiopterin by scavenging peroxynitrite-derived free radicals, thus preventing eNOS uncoupling. Inhibition of xanthine oxidase, lipoxygenase and NADPH oxidase, enzymes which produce reactive oxygen species: prevent NO breakdown.</td>
<td>Gryglewski et al. (1987)\textsuperscript{138} McCarty (2008)\textsuperscript{141} Mladenka et al. (2010)\textsuperscript{142}; Nijveldt et al. (2001)\textsuperscript{144}</td>
</tr>
<tr>
<td>General/nonspecific: other</td>
<td>Antioxidant effect: inhibit lipid oxidation Inhibition of xanthine oxidase, lipoxygenase and NADPH oxidase, enzymes which produce reactive oxygen species: other effects Chelation of metal ions: prevent formation of free radicals from metal catalysed reactions. Interaction with membrane lipids: could affect activity of membrane associated enzymes, ligand-receptor interactions, signal transduction and/or, ion/metabolite fluxes.</td>
<td>Fraga et al. (2010)\textsuperscript{135} Mladenka et al. (2010)\textsuperscript{142}; Nijveldt et al. (2001)\textsuperscript{144} Morel et al. (1993)\textsuperscript{143} Fraga et al. (2010)\textsuperscript{135}</td>
</tr>
<tr>
<td>Specific: NO related</td>
<td>Increased eNOS activity: enhanced NO production Increased eNOS expression: enhanced NO production Increased circulating nitrite: contributes to circulating NO pool and can be converted to NO when required 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.</td>
<td>Stoclet et al. (2004)\textsuperscript{147} Stoclet et al. (2004)\textsuperscript{147} Schroeter et al. (2006)\textsuperscript{137}; Balzer et al. (2008)\textsuperscript{139}; Heiss et al. (2003)\textsuperscript{146} Peri et al. (2005)\textsuperscript{145}; Takahama et al. (2002)\textsuperscript{148};</td>
</tr>
<tr>
<td>Specific: other</td>
<td>Enhanced prostacyclin production: vasodilation</td>
<td>Actis-Goretta et al. (2006)\textsuperscript{136}</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td></td>
<td>Enhanced endothelium-derived hyperpolarizing factor (EDHF) production: vasodilation</td>
<td>Stoclet et al. (2004)\textsuperscript{147}</td>
</tr>
<tr>
<td></td>
<td>Inhibition of endothelin-1 synthesis: vasodilation</td>
<td>Loke et al. (2008)\textsuperscript{140}</td>
</tr>
</tbody>
</table>

Inhibition of ACE activity: involved in regulation of renin-angiotensin system which could control NO production.

Takahama et al. (2010)\textsuperscript{149}, Volk et al. (2009)\textsuperscript{150}
Table 3: Observed adverse effects of flavonoids (summarised from 58,160,161).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antinutritional:</strong></td>
<td></td>
</tr>
<tr>
<td>Reduced glucose uptake</td>
<td>Observed in animal studies. Could have a protective effect by slowing absorption of glucose after a meal.</td>
</tr>
<tr>
<td>Impaired food utilisation</td>
<td>Proanthocyanidins interfere with protein utilisation in animal and human studies. Impaired lipid and carbohydrate metabolism observed with high flavonoid intakes in animal studies.</td>
</tr>
<tr>
<td>Impaired mineral absorption</td>
<td>Impairment of nonheme iron absorption observed in human studies. Heme iron absorption inhibited <em>in vitro</em> studies. Possible interaction between flavonoids and copper and manganese.</td>
</tr>
<tr>
<td>Impaired folate uptake</td>
<td>Observed <em>in vitro</em> studies. Little evidence of an effect in humans.</td>
</tr>
<tr>
<td>Vitamin C transport inhibition</td>
<td>Observed <em>in vitro</em> and animal studies.</td>
</tr>
<tr>
<td>Thyroid toxicity and goitrogenic activity</td>
<td>Observed <em>in vitro</em> and animal studies. No evidence of an effect in humans.</td>
</tr>
<tr>
<td>Drug interactions</td>
<td><em>in vitro</em> and animal studies suggest:</td>
</tr>
<tr>
<td></td>
<td>interactions with various cytochrome P450 monooxygenase (CYP) isoforms.</td>
</tr>
<tr>
<td></td>
<td>interactions with phase II enzymes.</td>
</tr>
<tr>
<td></td>
<td>interactions with drug transporters.</td>
</tr>
<tr>
<td>Genotoxicity/carcinogenicity</td>
<td>Observed <em>in vitro</em> and animal studies at high doses and concentrations.</td>
</tr>
<tr>
<td>Developmental effects</td>
<td>Possible association with infant acute myeloid leukemia (through effects on DNA topoisomerase II) but associated with a reduced risk for all leukemias.</td>
</tr>
</tbody>
</table>
Table 4: Summary of intervention studies examining both the acute and chronic effect of nitrate on blood pressure

<table>
<thead>
<tr>
<th>References</th>
<th>Nitrate Source</th>
<th>Acute/Chronic</th>
<th>Nitrate Dose</th>
<th>Subject characteristics and number</th>
<th>BP Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Webb et al. (2008)</td>
<td>Beetroot juice</td>
<td>Acute</td>
<td>1400 mg</td>
<td>Healthy M &amp; F (n=14)</td>
<td>SBP, DBP, MAP ↓</td>
</tr>
<tr>
<td>Lansley et al. (2011)</td>
<td>Acute</td>
<td>385 mg</td>
<td>Healthy M (n=9)</td>
<td>SBP ↓</td>
<td></td>
</tr>
<tr>
<td>Kapil et al. (2010)</td>
<td>Acute</td>
<td>350 mg</td>
<td>Healthy M &amp; F (n=14)</td>
<td>SBP ↓</td>
<td></td>
</tr>
<tr>
<td>Vanhatalo et al. (2010)</td>
<td>Acute</td>
<td>322 mg</td>
<td>Healthy M &amp; F (n=8)</td>
<td>SBP, DBP, MAP ↓</td>
<td></td>
</tr>
<tr>
<td>Kenjale et al. (2011)</td>
<td>Acute</td>
<td>560 mg</td>
<td>PAD M &amp; F (n=8)</td>
<td>DBP ↓</td>
<td></td>
</tr>
<tr>
<td>Coles and Clifton (2012)</td>
<td>Acute</td>
<td>465 mg</td>
<td>Healthy M &amp; F (n=30)</td>
<td>SBP ↓ M only</td>
<td></td>
</tr>
<tr>
<td>Kukadia et al. (2013)</td>
<td>Acute</td>
<td>400 mg</td>
<td>Healthy M &amp; F (n=9)</td>
<td>Central SBP ↓</td>
<td></td>
</tr>
<tr>
<td>Bailey et al. (2009)</td>
<td>Chronic (6 d)</td>
<td>340 mg</td>
<td>Healthy M (n=8)</td>
<td>SBP ↓</td>
<td></td>
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<tr>
<td>Bailey et al. (2010)</td>
<td>Chronic (6 d)</td>
<td>316 mg</td>
<td>Healthy M (n=7)</td>
<td>SBP, DBP, MAP ↓</td>
<td></td>
</tr>
<tr>
<td>Lansley et al. (2011)</td>
<td>Chronic (6 d)</td>
<td>385 mg</td>
<td>Healthy M (n=9)</td>
<td>SBP ↓</td>
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<tr>
<td>Vanhatalo et al. (2010)</td>
<td>Chronic (15 d)</td>
<td>322 mg</td>
<td>Healthy M &amp; F (n=8)</td>
<td>SBP, DBP, MAP ↓</td>
<td></td>
</tr>
<tr>
<td>Gilchrest et al. (2013)</td>
<td>Chronic (14 d)</td>
<td>465 mg</td>
<td>T2DM M &amp; F (n=27)</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Kelley et al. (2013)</td>
<td>Chronic (3 d)</td>
<td>595 mg</td>
<td>Healthy M &amp; F (n=12)</td>
<td>SBP, DBP ↓</td>
<td></td>
</tr>
<tr>
<td>Cermak et al. (2012)</td>
<td>Chronic (6 d)</td>
<td>500 mg</td>
<td>Healthy M (n=12)</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Hobbs et al. (2012)</td>
<td>Beetroot juice (dose response)</td>
<td>Acute</td>
<td>143 mg</td>
<td>Healthy M (n=4)</td>
<td>SBP, DBP ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>353 mg</td>
<td>Healthy M (n=4)</td>
<td>SBP, DBP ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>707 mg</td>
<td>Healthy M (n=4)</td>
<td>SBP, DBP ↓</td>
</tr>
<tr>
<td>Hobbs et al. (2012)</td>
<td>Red beetroot enriched bread</td>
<td>Acute</td>
<td>112 mg</td>
<td>Healthy M (n=5)</td>
<td>SBP ↓</td>
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<tr>
<td></td>
<td>White beetroot enriched bread</td>
<td></td>
<td>99 mg</td>
<td>Healthy M (n=5)</td>
<td>No effect</td>
</tr>
<tr>
<td>Study</td>
<td>Intervention</td>
<td>Duration</td>
<td>Dose</td>
<td>Participants</td>
<td>Blood Pressure Response</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------</td>
<td>----------</td>
<td>-----------------------------</td>
<td>------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Bondonno et al. (2012)</td>
<td>Spinach</td>
<td>Acute</td>
<td>182 mg</td>
<td>Healthy M &amp; F (n=30)</td>
<td>SBP ↓</td>
</tr>
<tr>
<td>Liu et al. (2013)</td>
<td></td>
<td></td>
<td>220 mg</td>
<td>Healthy M &amp; F (n=26)</td>
<td>SBP ↓</td>
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<tr>
<td>Sobko et al. (2010)</td>
<td>Japanese traditional diet</td>
<td>Chronic (10 d)</td>
<td>18.8 mg/kg (±1200 mg)</td>
<td>Healthy M &amp; F (n=25)</td>
<td>DBP ↓</td>
</tr>
<tr>
<td>Larsen et al. (2006)</td>
<td>Sodium nitrate</td>
<td>Chronic (3 d)</td>
<td>6.2 mg/kg (±400 mg)</td>
<td>Healthy M &amp; F (n=17)</td>
<td>DBP, MAP ↓</td>
</tr>
<tr>
<td>Larsen et al. (2007)</td>
<td></td>
<td>Chronic (3 d)</td>
<td>6.2 mg/kg (±400 mg)</td>
<td>Healthy M (n=9)</td>
<td>SBP, DBP ↓</td>
</tr>
<tr>
<td>Rammos et al. (2013)</td>
<td></td>
<td>Chronic (28 d)</td>
<td>12.75 mg/kg (±900 mg)</td>
<td>Elderly with moderate CV risk (n=11)</td>
<td>SBP ↓</td>
</tr>
<tr>
<td>Kapil et al. (2010)</td>
<td>Potassium nitrate</td>
<td>Acute</td>
<td>1488 mg</td>
<td>Healthy M &amp; F (n=21)</td>
<td>SBP, DBP ↓</td>
</tr>
<tr>
<td>Lidder et al. (2011)</td>
<td></td>
<td>Acute</td>
<td>1488 mg</td>
<td>Healthy M &amp; F (n=8)</td>
<td>No effect</td>
</tr>
<tr>
<td>Bahra et al. (2012)</td>
<td></td>
<td>Acute</td>
<td>500 mg</td>
<td>Healthy M &amp; F (n=14)</td>
<td>SBP ↓</td>
</tr>
</tbody>
</table>
Table 5: Summary of intervention studies examining both the acute and chronic effect of nitrate on measures of FMD

<table>
<thead>
<tr>
<th>References</th>
<th>Nitrate Source</th>
<th>Acute/Chronic</th>
<th>Nitrate Dose</th>
<th>Subject characteristics</th>
<th>FMD Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Webb et al. (2008)</td>
<td>Beetroot juice</td>
<td>Acute</td>
<td>1400 mg</td>
<td>Healthy M &amp; F (n=14)</td>
<td>FMD↑</td>
</tr>
<tr>
<td>Kapil et al. (2010)</td>
<td>Potassium nitrate</td>
<td>Acute</td>
<td>1488 mg</td>
<td>Healthy M &amp; F (n=12)</td>
<td>FMD↑</td>
</tr>
<tr>
<td>Bondonno et al. (2012)</td>
<td>Beetroot juice</td>
<td>Acute</td>
<td>350 mg</td>
<td>Healthy M &amp; F (n=12)</td>
<td>FMD↑</td>
</tr>
<tr>
<td>Heiss et al. (2012)</td>
<td>Spinach</td>
<td>Acute</td>
<td>182 mg</td>
<td>Healthy M &amp; F (n=30)</td>
<td>FMD↑</td>
</tr>
<tr>
<td>Bahra et al. (2012)</td>
<td>Sodium nitrate</td>
<td>Acute</td>
<td>1000 mg</td>
<td>Healthy M &amp; F (n=10)</td>
<td>FMD↑</td>
</tr>
<tr>
<td>Gilchrest et al. (2013)</td>
<td>Beetroot juice</td>
<td>Acute</td>
<td>465 mg</td>
<td>T2DM M &amp; F (n=27)</td>
<td>FMD no effect</td>
</tr>
<tr>
<td>Rammos et al. (2013)</td>
<td>Sodium nitrate</td>
<td>Chronic (28 d)</td>
<td>12.75 mg/kg (±900 mg)</td>
<td>Elderly with moderate CV risk (n=11)</td>
<td>FMD↑</td>
</tr>
<tr>
<td>Joris et al. (2013)</td>
<td>Beetroot juice</td>
<td>Acute</td>
<td>500 mg</td>
<td>Healthy but overweight BMI 28-35 kg/m² (n=20)</td>
<td>FMD↑</td>
</tr>
</tbody>
</table>