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ROLE OF FLAVONOIDS AND NITRATES IN CARDIOVASCULAR HEALTH

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Running title: Flavonoids, nitrates and CVD

Key words: platelet function; blood pressure; dietary nitrate; flavonoids; vascular function;

Abbreviations: CVD, cardiovascular disease; CHD, coronary heart disease; CI, confidence interval; DASH, dietary approach to stop hypertension; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase, FMD, flow mediated dilation; GLUT, glucose transporter ; HDL-C, high-density lipoprotein cholesterol; iNOS, inducible nitric oxide synthase; LDI, laser Doppler imaging with iontophoresis; LDL-C, low-density lipoprotein cholesterol; MAP, Mitogen-Activated Protein Kinase; MI, myocardial infarction; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; PLC γ 2, phospholipase C γ 2; RR, relative risk; RCT, randomised control trial; SGLUT1, sodium-dependent glucose transporter 1; TAG, triacylglycerol; SYK, spleen tyrosine kinase.

1 Abstract

2 Cardiovascular diseases (CVD) remain the leading cause of death globally. Effective dietary
3 strategies for their prevention are of high priority. Increasing evidence suggests that
4 phytochemicals, particularly dietary flavonoids and nitrates, are key modulators of CVD risk
5 reduction through impact on multiple risk factors. The aim of this review is to explore the evidence
6 for the impact of flavonoid- and nitrate-rich foods and supplements on CVD risk, with specific
7 reference to their importance as mediators of vascular health and platelet function. There is
8 accumulating evidence to support benefits of dietary flavonoids on cardiovascular health. Dose-
9 dependent recovery of endothelial function and lowering of blood pressure have been reported for
10 the flavanol (-)-epicatechin, found in cocoa, apples and tea, through production and availability of
11 endothelial nitric oxide (NO). Furthermore, flavonoids, including quercetin and its metabolites,
12 reduce *in vitro* and *ex vivo* platelet function via inhibition of phosphorylation-dependent cellular
13 signalling pathways, though further *in vivo* studies are required to substantiate these mechanistic
14 effects. Hypotensive effects of dietary nitrates have been consistently reported in healthy subjects in
15 acute and chronic settings, though there is less evidence for these effects in patient groups.
16 Proposed mechanisms of actions include endothelial-independent NO availability, which is
17 dependent on the entero-salivary circulation and microbial conversion of dietary nitrate to nitrite in
18 the mouth. In conclusion, flavonoid and nitrate-rich foods show promising effects on vascular
19 function, yet refinement of qualitative dietary guidelines will require confirmation of this evidence,
20 together with further randomly controlled trials for determination of effective doses.

21

22

23

24 **Introduction**

25 Cardiovascular diseases (CVD) are the leading cause of mortality globally, accounting for around
26 31 % of deaths each year⁽¹⁾. In the UK CVD was the second most common cause of death in 2014,
27 responsible for 27 % of all mortalities⁽²⁾. There are several recognised risk factors for CVD
28 including raised serum low-density lipoprotein (LDL)-cholesterol and triacylglycerol (TAG), low
29 serum high-density lipoprotein (HDL)-cholesterol, elevated blood pressure, diabetes and obesity
30 many of which can be modified by the lifestyle choices, including diet⁽³⁾. Epidemiological data
31 from the 1970's indicated that coronary heart disease (CHD) rates were higher in countries with low
32 fruit and vegetable consumption⁽⁴⁾. This has been supported by a number of more recent studies that
33 have shown that dietary patterns rich in fruit and vegetables are associated with reduced rates of
34 CHD, stroke and CVD mortality^(5; 6; 7; 8; 9; 10). Some researchers have attempted to identify the types
35 of fruits and vegetables responsible for the reduced risk of CVD. Joshipura *et al* in 2001 showed
36 that people in the highest quintile of fruit and vegetable intake had a 20% lower relative risk (RR)
37 for CHD compared with those in the lowest quintile of intake. In addition, each 1 serving per day
38 increase in fruit and vegetable intake was associated with a 4 % lower risk of CHD. They also
39 found that vitamin C-rich fruits (6 % lower RR per 1 serving/day increase) and particularly green
40 leafy vegetables (23 % lower RR per 1 serving/day increase) had the largest protective effects⁽⁵⁾.

41 Fruits and vegetables are a rich source of phytochemicals such as flavonoids and dietary nitrates,
42 which have been shown to independently exert a number of health effects and could be responsible,
43 at least in part, for the apparent protective effects of fruit and vegetable consumption. The aim of
44 this review is to provide a brief overview of evidence related to the effects of flavonoids and dietary
45 nitrates on cardiovascular health with particular reference to vascular and platelet function.

46

47 **Dietary flavonoids**

48 The main categories of phytochemicals are polyphenols, which include flavonoids, terpenoids,
49 nitrogen-containing alkaloids and sulphur-containing compounds. Flavonoids are produced by
50 plants as secondary metabolites and have biological roles in plant pigmentation, flavour, growth,
51 reproduction, predator and pathogen resistance⁽¹¹⁾. They are present in a variety of foods including
52 vegetables, fruits, nuts, grains, red wine and chocolate, in concentrations that vary due to a number
53 of factors, including environmental stress, such as UV exposure⁽¹²⁾. Flavonoids consist of two
54 benzene rings linked by a 3-carbon chain (C6-C3-C6) as shown in **Figure 1**. The flavonoid classes
55 differ due to the C ring structural differences, number of phenolic hydroxyl groups and their
56 substitutions, and are commonly divided into seven structural subclasses namely: isoflavones,
57 flavanols or catechins, flavanonols, flavonols, flavanones, flavones, anthocyanins and

58 anthocyanidins⁽¹³⁾ (**Figure 2**). The small structural variations between subclasses are related to
59 considerable differences in biological functions.

60

61 *Absorption and Metabolism of flavonoids*

62 Flavonoids are commonly found in the diet as conjugated esters, glycosides or polymers, which
63 have limited bioavailability, requiring intestinal enzyme hydrolysis or colonic microbiota
64 fermentation before absorption into the circulation. Aglycones formed in the intestine by cleavage
65 of flavonoid side chains can enter the epithelial cell by passive diffusion⁽¹⁴⁾. However polar
66 glucosides can be actively transported into epithelial cells via the sodium-dependent glucose
67 transporter 1 (SGLUT1), where they are hydrolysed by intracellular enzymes to the aglycone⁽¹⁵⁾.
68 The importance of the latter absorption route is unclear, but glycosylated flavonoids and aglycones
69 have been shown to inhibit the SGLUT1 transporter, potentially reducing dietary glucose
70 absorption⁽¹⁶⁾. Before transport to the circulation, the aglycones also undergo further metabolism
71 (Phase II) and conjugation including glucuronidation, methylation or sulfation. Efflux of the
72 metabolites back into the intestine also occurs via transporters including multidrug resistance
73 protein and P-glycoprotein and the glucose transporter GLUT2⁽¹⁷⁾. Further Phase II metabolism
74 occurs in the liver via portal vein transportation and further recycling into the intestinal lumen via
75 the enterohepatic recirculation in bile⁽¹⁸⁾. Some flavonoids, particularly polyphenol sugar
76 conjugates, pass unabsorbed into the colon and are associated with marked modulation of the
77 colonic gut microbiota⁽¹⁹⁾ and the production of principally small phenolic acid and aromatic
78 catabolites, which are subsequently absorbed into the circulation⁽²⁰⁾. These metabolites can be
79 subjected to further metabolism in the liver before they are efficiently excreted in the urine in
80 quantities far higher than those that entered the circulation via the intestine⁽²¹⁾. Due to the extensive
81 metabolism and rapid excretion, plasma concentrations do not reflect quantitative absorption and
82 total urinary metabolite excretion can be a more valuable biomarker of intake. Evidence for tissue
83 accumulation of polyphenols and their metabolites is very limited and while this can't be ruled out,
84 it is believed that frequent ingestion of flavonoid-rich foods is required to maintain constant
85 circulating levels (see review ⁽²²⁾). Detailed studies using stable isotopes have allowed the
86 determination of metabolic pathways of certain polyphenol subclasses. Anthocyanins, found in
87 foods such as berries, are reported to have low bioavailability, but recent data have shown they are
88 extensively metabolised to a diverse range of metabolites, which has highlighted a previous
89 underestimation of anthocyanin absorption and metabolism⁽²³⁾.

90

91 *Flavonoid intake and CVD risk: Epidemiological studies*

92 Epidemiological studies have produced strong evidence for the negative association between high
93 fruit and vegetable consumption and cardiovascular disease mortality^(24; 25). Yet it is difficult to
94 identify the specific mediator(s) of health due to the numerous potential bioactive compounds
95 present in fruits and vegetables. Observational studies suggest that intakes of flavonoids are
96 associated with a decreased risk of CVD, although the findings are not entirely consistent, because
97 of variation in population studied, dose and specific flavonoid consumed. Data from a large post-
98 menopausal cohort identified a negative association between flavonoid-rich diets and CVD
99 mortality⁽²⁶⁾. Further evidence showed intakes of flavanol- and procyanidin-rich foods were
100 associated with decreased risk of chronic non-communicable diseases particularly CVD^(27; 28). In
101 2005, Arts and Hollman collated data from 15 prospective cohort studies, of these 13 provided
102 evidence for a positive association between dietary flavanols, procyanidins, flavones and flavanones
103 and CVD health, with a reduction of CVD mortality of approximately 65 %⁽²⁹⁾. Systematic
104 reviews^(30; 31; 32) that have focused on flavonol intake have reported inconsistent findings including
105 an inverse association between high flavonol intake and CHD or stroke mortality^(31; 32) compared
106 with no association between flavonol intake and CHD risk⁽³⁰⁾. However, a more recent
107 comprehensive systematic review and meta-analysis of 14 studies identified that intakes of
108 anthocyanidins, proanthocyanidins, flavones, flavanones and flavan-3-ols were associated with
109 lower CVD RR of 11 %, 10 %, 12 %, 12 % and 13 % respectively when comparing the highest and
110 lowest categories of intake; with a 5 % lower RR for CVD for every 10mg/d increment in flavonol
111 intake⁽³³⁾.

112

113 An inverse association between flavanol intake and CVD mortality was initially identified in the
114 Iowa women health study, which followed 34,489 women, free of CVD at study inclusion ⁽³⁴⁾ while,
115 a subsequent follow-up found no association between reduced CVD risk and flavanol intake,
116 instead an association with procyanidin intake⁽²⁶⁾. These seemingly contrasting findings were due to
117 the different ways the data from chocolate and seeded grapes were categorized in the dietary
118 assessment, and emphasises the importance of standardisation of dietary assessment, and the
119 possible benefits of using biomarkers of intake. Evidence from prospective cohort studies generally
120 supports the hypothesis that a greater intake of dietary flavonoids is associated with a lower risk of
121 CVD, although there are inconsistencies in potential benefit. Further supportive evidence from
122 well-performed randomly controlled dietary intervention studies is required to establish a direct
123 relationship between different flavonoid sub-groups and CVD risk.

124

125 *Chronic and acute effects of flavonoid intake on micro- and macrovascular function*

126 The vascular endothelium plays a key role in the regulation of vascular homeostasis, and alterations

127 in endothelial function contribute to the pathogenesis and clinical expression of CVD⁽³⁵⁾. Many
128 factors impact adversely on the endothelium, these include diabetes mellitus, smoking, physical
129 inactivity, aging, hypertension, systemic inflammation, dyslipidaemia and insulin resistance, with
130 diet being key in modulating endothelial function^(36; 37). Prospective cohort studies have supported
131 the association between endothelial function and an increased risk of CVD events and have
132 identified the latter as a valuable holistic surrogate marker of CVD risk^(38; 39). Endothelial
133 dysfunction has been associated with the development of atherosclerosis and CVD⁽⁴⁰⁾ and is most
134 commonly measured in the brachial artery by flow mediated dilatation (FMD), which uses non-
135 invasive ultrasound before and after increasing shear stress by reactive hyperaemia, with the degree
136 of dilation reflecting arterial NO release. Another commonly used technique is laser Doppler
137 imaging with iontophoresis (LDI), which measures the endothelial function of the peripheral
138 microcirculation. The degree of endothelial dysfunction occurring in the microcirculation has been
139 shown to be proportional to that occurring in the coronary arteries⁽⁴¹⁾. This technique measures the
140 response of cutaneous blood vessels to transdermal delivery of two contrasting vasoactive agents:
141 acetylcholine (endothelium-dependent vasodilator) and sodium nitroprusside (endothelium-
142 independent vasodilator) by iontophoresis. A reduced local vasodilatory response to acetylcholine is
143 associated with endothelial dysfunction⁽⁴²⁾.

144

145 Consumption of fruits rich in anthocyanins and proanthocyanidins in the form of purple grape juice
146 or grape seed extract (for between 14-28 days) significantly increased FMD in volunteers with
147 angiographically documented CHD or above average vascular risk⁽⁴³⁾. Furthermore, consumption of
148 pomegranate, containing tannins and anthocyanins, for a period of 90 days to 3 years resulted in
149 improvements in carotid intima thickness (CIMT), a measure of the extent of atherosclerosis in
150 the carotid artery, in those with increased CVD risk⁽⁴³⁾. The FLAVURS study investigated the dose-
151 dependent effect (+2, +4 and +6 additional portions/d) of flavonoid-rich and flavonoid-poor fruits
152 and vegetables compared to habitual diet, on microvascular reactivity, determined by LDI, and
153 other CVD risk markers. After two additional portions of flavonoid-rich fruits and vegetables,
154 equivalent to an estimated increase in total dietary flavonoids from 36 ± 5 to 140 ± 14 mg/d⁽⁴⁴⁾, a
155 significant increase in endothelium-dependent microvascular reactivity was observed in men. In
156 addition, reduced C-reactive protein, E-selectin and vascular cell adhesion molecule, and increased
157 plasma NOx was observed with four additional flavonoid-rich portions, compared to the control and
158 low-flavonoid intervention⁽⁴⁵⁾. This data supports vascular improvements reported in a previous
159 study investigating a similar single dose of flavonoid-rich foods⁽⁴⁶⁾. Identification of the specific
160 flavonoid bioactive is not possible in studies that include a variety of foods, yet these data

161 demonstrate that dietary relevant doses of total flavonoids can contribute to vascular health and
162 could be considered as useful strategies for CVD risk factor reduction.

163

164 The majority of the population is in a postprandial state for most of the day and it is recognized that
165 acute physiological responses to meals are a major contributor to overall CVD risk. Flavonoid-rich
166 foods have been implicated in modulating postprandial responses. For example blueberries are a
167 rich source of flavonoids, particularly anthocyanin, flavanol oligomer, and chlorogenic acid⁽⁴⁷⁾.

168 Acute improvements in vascular function, measured by FMD, were observed in healthy men in a
169 time- and dose-dependent manner (up to a concentration of 766mg total polyphenols)⁽⁴⁸⁾ with little
170 observed effect of processing⁽⁴⁷⁾. These beneficial effects on postprandial vascular reactivity are not
171 confined to blueberries, as a mixed fruit puree containing, 457 mg (-)-epicatechin increased
172 microvascular reactivity and plasma NO_x⁽⁴⁹⁾. Although the fruit puree contained varied flavonoids,
173 the potential vascular benefits of (-)-epicatechin is supported by a meta-analysis of six randomly
174 controlled trials which found that 70-177 mg (-)-epicatechin, from cocoa or chocolate sources
175 significantly increased postprandial FMD by 3.99 % at 90-149 min post ingestion⁽⁵⁰⁾. These data
176 indicate that different classes of flavonoids in the form of foods can significantly improve
177 postprandial vascular function and possible CVD risk.

178

179 Flavanols, as a subgroup of flavonoids, have been extensively studied and increasing evidence has
180 shown that higher intake of flavanol-rich foods improve arterial function in numerous groups
181 including those at risk for CVD, with established CVD⁽⁵¹⁾ and more recently healthy young and
182 aging individuals⁽⁵²⁾. The mechanisms of action are not totally understood, but causality between
183 intake and an improvement in arterial function has been demonstrated⁽⁵³⁾. The important dietary
184 flavanol, (-)-epicatechin, is naturally present in high concentrations in cocoa, apples and tea and a
185 number of systematic reviews and meta-analyses, including a recent study of 42 randomised
186 controlled human dietary intervention studies on supplemental and flavan-3-ols-rich chocolate and
187 cocoa, reported significant acute and chronic (up to 18 weeks) dose-dependent cardiovascular
188 benefits, including recovery of endothelial function, lowering of blood pressure and some
189 improvements in insulin sensitivity and serum lipids^(50; 54; 55). Furthermore green and black tea (rich
190 in (-)-epicatechin) was also reported to reduce blood pressure and LDL-cholesterol in a systematic
191 review and meta-analysis of a small number of studies, but these findings need confirmation in
192 long-term trials, with low risk of bias⁽⁵⁶⁾. The extensive studies into the vascular effects of (-)-
193 epicatechin and their impact on other CVD risk markers has prompted some to propose specific
194 dietary recommendation for these flavonoids and a broader recommendation on flavanol-rich fruits

195 and vegetables for CVD risk reduction, although further evidence may be required before specific
196 recommendations are considered.⁽⁵⁷⁾

197

198 *Possible mechanisms of flavonoids and vascular effects*

199 Despite the high antioxidant potential of a number of classes of flavonoids, there is limited evidence
200 to support this mechanism of action due to the low plasma concentrations of flavonoids compared
201 with other endogenous or exogenous antioxidants⁽⁵⁸⁾. Many of the vascular effects of flavonoids
202 have been associated with molecular signalling cascades and related regulation of cellular function.
203 One of the potential mechanisms of action is the association between flavonoid, particularly (-)-
204 epicatechin, and prolonged, augmented nitric oxide (NO) synthesis, the primary modulator of
205 vascular dilation⁽⁵³⁾. NO production from L-arginine is regulated by three nitric oxide synthase
206 (NOS) enzymes: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) with
207 lower production and/or availability of NO as the main effect on endothelial dysfunction. Several *in*
208 *vitro* and human studies have reported potent vasorelaxant activity of certain flavonoids related to
209 activation of eNOS^(53; 59). A common polymorphism in the eNOS gene is the Glu298Asp single-
210 nucleotide polymorphism that modifies its coding sequence, replacing a glutamate residue at
211 position 298 with an aspartate residue. This polymorphism has been linked to increased risk of
212 cardiovascular events putatively through reduced NO production by eNOS^(60; 61). Interestingly, in a
213 small acute randomized control study a significant genotype interaction with endothelium-
214 dependent microvascular dilation was observed after consumption of fruit and vegetable puree
215 containing 456 mg (-)-epicatechin. Wild-type, GG, participants (non-risk group) showed an
216 increased endothelial vasodilation at 180 min compared to control, with no effect in T allele
217 carriers. This supports the importance of (-)-epicatechin in eNOS activation and NO availability,
218 with little vascular effect of (-)-epicatechin in those with impaired eNOS function. This nutrient-
219 gene interaction may explain in part, the large variation in individual vascular responses to
220 flavonoid consumption, but requires further confirmatory studies⁽⁶²⁾.

221

222 Flavonoids have also been reported to modulate xanthine oxidase activity, resulting in decreased
223 oxidative injury and consequential increased NO⁽⁶³⁾. The vascular effects induced by phenolics may
224 also be mediated by the inhibition of Ca²⁺ channels and/or the blockage of the protein kinase C-
225 mediated contractile mechanism, as has been observed for caffeic acid phenyl ester and sodium
226 ferulate, respectively⁽⁶⁴⁾. Furthermore, benefits may be mechanistically linked to the actions of
227 circulating phenolic metabolites on inhibition of neutrophil nicotinamide adenine dinucleotide
228 phosphate (NADPH) oxidase activity, which prevents NO degradation and increases its
229 availability⁽⁴⁸⁾. More recently a possible role of flavonoid promotion of endothelium-derived

230 hyperpolarizing factor (EDHF) in vasodilation, which induces hyperpolarization, thus leading to
231 dilation of the vascular smooth muscle cell has been identified⁽⁶⁵⁾. In summary there are multiple
232 potential mechanisms by which flavonoids and their metabolites can modulate vascular function⁽⁶⁶⁾
233 and these may act in an additive or synergistic manner. It is evident that dietary relevant doses of
234 flavonoids are associated with vascular benefit with varied proposed modulating mechanisms that
235 require elucidation in further studies.

236

237 *Flavonoids and platelet aggregation*

238 Platelets are small nucleated cell fragments that are produced by megakaryocytes in the bone
239 marrow^(67; 68) and play a critical role in haemostasis through formation of aggregates over arterial
240 wall injuries⁽⁶⁹⁾. When platelet activation becomes impaired, thrombosis can occur, a
241 pathophysiological condition which can lead to blockage of coronary arteries or impaired blood
242 supply to the brain, leading to events such as myocardial infarction (MI) or stroke⁽⁷⁰⁾. Many studies
243 have previously shown the ability of flavonoids to inhibit platelet function^(71; 72; 73). Quercetin is
244 found in many foods such as apples, onions, tea and wine, and present in significant quantities in
245 many diets⁽⁷⁴⁾. Further understanding of how quercetin modulates platelet function is of relevance
246 to establish a mechanistic link between flavonols and CVD risk.

247 Hubbard *et al.*⁽⁷⁵⁾ observed a significant inhibition of *ex vivo* platelet aggregation after ingestion
248 of quercetin-4'-O- β -D-glucoside at a dose of 150 mg and 300 mg, with peak quercetin metabolite
249 concentrations of 4.66 μ M and 9.72 μ M respectively. This data was supported by a further small
250 human study which reported at peak plasma quercetin metabolite concentrations of 2.59 μ M and
251 significant inhibition of *ex vivo* collagen-stimulated platelet aggregation 60 and 240 min after
252 consumption of a high-quercetin onion soup rich in quercetin glucosides (68.8 mg total quercetin)
253 compared with a matched low quercetin onion control (4.1 mg total quercetin)⁽⁷⁶⁾. Inhibition of
254 Spleen Tyrosine Kinase (SYK) and Phospholipase C γ 2 (PLC γ 2), two key platelet proteins involved
255 with the collagen-stimulated signalling pathway were also observed, and confirms this as one
256 potential mechanism of action. These data are in agreement with previous *in vitro* studies displaying
257 the ability of quercetin to inhibit collagen, ADP and thrombin-stimulated platelet aggregation, as
258 well as inhibiting collagen-stimulated Mitogen-Activated Protein (MAP) kinases and
259 Phosphoinositide 3-kinase (PI3K) phosphorylation^(77; 78).

260 Flavonoids undergo significant endogenous metabolism and it is important to determine the
261 bioactivity of metabolites as well as the aglycones by understanding structure-activity relationships
262 and how functional groups affect platelet function. Anti-platelet effects of tamarixetin, quercetin-3-
263 sulphate and quercetin-3-glucuronide, as well as the structurally distinct flavonoids apigenin and
264 catechin, quercetin and its plasma metabolites were determined. Quercetin and apigenin

265 significantly inhibited collagen-stimulated platelet aggregation and 5-HT secretion with similar
266 potency, and logIC₅₀ values for inhibition of aggregation of -5.17 (\pm 0.04) and -5.31 (\pm 0.04),
267 respectively⁽⁷⁹⁾. Flavones (such as apigenin) are characterized by a non-hydroxylated C-ring,
268 whereas the C-ring of flavonols (e.g. quercetin) contain a C-3 hydroxyl group (**Figure 1**). Catechin
269 was less effective, with an inhibitory potency two orders of magnitude lower than quercetin,
270 suggesting that *in vivo*, metabolites of quercetin and apigenin may be more relevant in the inhibition
271 of platelet function. Flavan-3-ols such as catechin possess a non-planar, C-3 hydroxylated C ring,
272 which is not substituted with a C-4 carbonyl group (as is found in flavonols). Quercetin-3-sulphate
273 and tamarixetin (a methylated quercetin metabolite) were less potent than quercetin, with a
274 reduction from high to moderate potency upon addition of a C-4' methyl or C-3'-sulphate group,
275 but at concentrations above 20 μ M, all achieved substantial inhibition of platelet aggregation and 5-
276 HT release. Quercetin-3-glucuronide caused much lower levels of inhibition, providing evidence for
277 reduced potency upon glucuronidation of the C ring. Jasuja *et. al* have shown quercetin-3-
278 glucuronide to potently inhibit protein disulphide isomerase (PDI), an oxidoreductase important in
279 thrombus formation⁽⁸⁰⁾. X-ray crystallographic analyses of flavonoid-kinase complexes have shown
280 that flavonoid ring systems and the hydroxyl groups are important features for kinase binding ^{(81) (82)}
281 supporting the evidence for structure-specific effects on platelet function. Taken together, this
282 evidence shows the importance of understanding the structural differences of flavonoids, and how
283 specific functional groups on polyphenols can lead to enhanced or reduced effects in different
284 stages of haemostasis and thrombosis. This evidence may also facilitate the design of small-
285 molecular inhibitors and inform specific dietary advice.

286

287 In summary, flavonoids are generally poorly absorbed and substantially metabolised to aid rapid
288 elimination. Many flavonoid subgroups reach the colon in their native state, and are fermented by
289 the microbiota, which produces small phenolic metabolites with potential bioactivity after
290 absorption. CVD risk reduction from high fruit and vegetable intake may be due, in part, to benefits
291 from flavonoid ingestion. In particular, (-)-epicatechin, a key flavanol, has been causally linked
292 with increased arterial endothelial-dependent dilation measured by FMD, with a putative increase in
293 NO bioavailability. Other potential mechanisms of action include modulation of NADPH oxidase
294 activity and reduction of NO degradation. Furthermore, flavonoids, particularly quercetin and its
295 metabolites, reduce *in vitro* and *ex vivo* platelet function, possibly via inhibiting phosphorylation in
296 cell signalling cascades. Further research will be required to determine the biological effects of
297 flavonoid subgroups *in vivo*, and the minimal effective dose of these compounds before it is
298 possible to make any specific dietary recommendations.

299

300

301 **Inorganic nitrate and nitrite**

302 Inorganic nitrate and nitrite were previously considered largely inactive by products of the
303 oxidation of NO endogenously. However, emerging evidence suggest these anions are important
304 storage forms of NO, which can be reduced to bioactive NO under certain conditions. Nitrate is
305 particularly abundant in vegetables such as beetroot and green leafy varieties (spinach, lettuce,
306 rocket) where it is absorbed from the soil and transported to the leaf where it accumulates. Nitrate is
307 important for plant function and is the main growth-limiting factor. In UK diets, estimates from
308 1997 suggest that the average nitrate intake is approximately 52 mg/day, with vegetables being the
309 main source of nitrate, contributing around 70 % of daily intakes with the remaining nitrate derived
310 from drinking water⁽⁸³⁾.

311 The consumption of inorganic nitrate either from dietary or supplemental sources have been
312 shown to exert a number of important vascular effects such as blood pressure lowering, protection
313 against ischemia-reperfusion injury, inhibiting platelet aggregation, preserving or improving
314 endothelial dysfunction and enhancing exercise performance⁽⁸⁴⁾.

315

316 *The nitrate-nitrite-NO pathway*

317 The continuous generation of NO from L-arginine by the enzymatic action of eNOS in the presence
318 of oxygen within endothelial cells is important for maintenance of vascular homeostasis. Indeed
319 reduced production or bioavailability of NO is associated with a number of cardiovascular and
320 metabolic disorders⁽⁸⁵⁾. The nitrate-nitrite-NO pathway is a NOS and oxygen independent pathway
321 for the generation of bioactive NO, and is an important alternative pathway for NO production,
322 particularly during periods of hypoxia⁽⁸⁶⁾. Ingested nitrate, obtained mainly from green leafy
323 vegetables and beetroot, is readily absorbed in the upper part of the gastrointestinal tract where it
324 mixes with NO produced from NOS ⁽⁸⁷⁾.

325

326 Ingested nitrate peaks after approximately 1 hour⁽⁸⁸⁾ and remains elevated for up to 5-6 hours post
327 ingestion. The majority of ingested nitrate (65-75 %) is excreted in urine with a very small
328 proportion of nitrate (<1 %) reaching the large bowel, which is excreted in the faeces⁽⁸⁹⁾. The
329 remaining nitrate is reabsorbed by the salivary glands and concentrated up to 20-fold, reaching
330 concentrations of 10 mM in the saliva⁽⁹⁰⁾. Salivary nitrate is converted to nitrite via a two-electron
331 reduction, a reaction that mammalian cells are unable to perform, during anaerobic respiration by
332 nitrate reductases produced by facultative and obligate anaerobic commensal oral bacteria^(86; 91).
333 The importance of oral bacteria in the nitrate-nitrite-NO pathway has been demonstrated in a
334 number of studies^(88; 92; 93). When the nitrite rich saliva reaches the acidic environment of the

335 stomach some of it reacts to form nitrous acid, which further decomposes to NO and other reactive
336 nitrogen oxides⁽⁹⁴⁾. The remaining nitrite (approximately 95 %) is absorbed into the circulation⁽⁹⁵⁾
337 where it forms NO via the action of a number of different nitrite reductases, which have selective
338 activity under oxygen/hypoxic/ischaemic conditions. These include haemoglobin⁽⁹⁶⁾, myoglobin⁽⁹⁷⁾,
339 cytoglobin and neuroglobin⁽⁹⁸⁾, xanthine oxidoreductase⁽⁹⁹⁾, aldehyde oxidase⁽¹⁰⁰⁾, aldehyde
340 dehydrogenase type 2⁽¹⁰¹⁾, eNOS⁽⁹⁹⁾, cytochrome P450⁽¹⁰²⁾ and the mitochondrial electron transport
341 chain⁽¹⁰³⁾. It is likely that the majority of the cardioprotective effects observed from dietary nitrate
342 consumption are via the conversion of nitrite to NO in blood and tissues.

343

344 **Vascular effects of dietary nitrate and nitrite**

345 Beneficial effects of nitrate consumption on vascular related function was first identified by Larsen
346 *et al.*⁽¹⁰⁴⁾, who showed that supplementation of healthy humans for three days with sodium nitrate
347 reduced blood pressure. Since then, a number of studies have shown that dietary nitrate-rich
348 vegetable sources such as beetroot juice, spinach, rocket and breads also lower blood pressure and
349 vascular function in healthy subjects^(88; 105; 106; 107).

350

351 *Endothelial dysfunction*

352 A hallmark of endothelial dysfunction is the reduced bioavailability of NO, either through reduced
353 eNOS activity or expression, or via increased NO consumption by free radicals and reactive oxygen
354 species⁽¹⁰⁸⁾ as discussed above. It has been shown that consumption of 500 mL beetroot juice
355 containing 23 mmol nitrate reversed the deleterious effects of a mild ischaemia-reperfusion injury
356 to the forearms of healthy subjects and preserved the FMD response, whereas the response was
357 reduced by 60 % in the control subjects^(88; 93). Hobbs *et al.*⁽¹⁰⁵⁾ found that consumption of bread
358 enriched with beetroot increased endothelium-independent blood flow in healthy subjects measured
359 by LDI. In healthy overweight and slightly obese subjects consumption of 140 mL beetroot juice
360 (500 mg nitrate) or control alongside a mixed meal (57 g fat) attenuated postprandial impairment of
361 FMD⁽¹⁰⁹⁾. More recently daily consumption of dietary nitrate in the form of beetroot juice over a 6-
362 week period resulted in a 1.1 % increase in the FMD response compared with a 0.3 % worsening in
363 the control group⁽¹¹⁰⁾. However, not all studies have found a beneficial effect of dietary nitrate on
364 endothelial function, with no effects of 250 mL beetroot juice (7.5 mmol nitrate) on FMD response
365 in patients with type 2 diabetes⁽¹¹¹⁾. Furthermore, supplemental potassium nitrate consumption (8
366 mmol nitrate) did not affect FMD response in healthy subjects, although a significant reduction (0.3
367 m/s) in pulse wave velocity and SBP (4 mm Hg) at 3 h compared with the potassium chloride
368 control was reported. This suggests that although inorganic nitrate did not alter endothelial function,
369 it did appear to increase blood flow in combination with reductions in BP.

370

371 Organic and inorganic nitrate/nitrites are both effective in vascular health, yet it has been proposed
372 that inorganic dietary nitrate may be a more appropriate choice for vascular modulation than
373 organic nitrate supplements⁽¹¹²⁾. The enterosalivary circulation is key for the effects of inorganic
374 nitrate and prevents a sudden effect, or toxic circulating concentrations of nitrite, in addition to
375 prolonging the vascular effects. In contrast, supplemental organic nitrate, which does not require the
376 enterosalivary circulation for absorption, has rapid pharmacodynamic responses, causing potent
377 acute effects, immediate vasodilation, and in chronic use considerably limited by the development
378 of tolerance and endothelial dysfunction. The more subtle and controlled effects of inorganic nitrate
379 may compensate for diminished endothelial function, and also has no reported tolerance. Therefore,
380 with the increasing recognition of the limitations of organic nitrate supplementation, and continuing
381 discovery of beneficial effects of inorganic nitrate/nitrite, dietary inorganic forms may prove to be
382 the optimum strategy for vascular health⁽¹¹²⁾.

383

384 *eNOS Glu298Asp polymorphism and nitrate interactions*

385 Variation in response to nitrates could be due to genetic polymorphisms. Healthy men
386 retrospectively genotyped for the Glu298Asp polymorphism (7 GG and 7 T carriers), showed a
387 differential postprandial BP response after consumption of beetroot-enriched bread compared with
388 the control bread. A significantly lower DBP in the T carriers was observed with a concomitant
389 tendency for higher plasma NO_x concentration. Despite the small study size these data suggests that
390 carriers of the T allele, which limits endogenous NO production from endothelial eNOS⁽¹¹³⁾, were
391 more responsive to dietary nitrate. Cross-talk between NOS-dependent pathway and the nitrate-
392 nitrite-NO pathway in control of vascular NO homeostasis could be a possible explanation for these
393 observations⁽¹¹⁴⁾, although future suitably powered studies are needed to confirm these findings.
394 This nutrient-gene interaction is in contrast to that demonstrated for the same eNOS polymorphism
395 and dietary flavonoids (described above), and confirms the differential proposed mechanisms by
396 which flavonoids and nitrates impact on NO availability and vascular function.

397

398 *Blood pressure*

399 Dietary nitrate has been shown to reduced SBP and/or DBP, and increase circulating nitrate/nitrite
400 (see review⁽¹¹⁵⁾). These findings are supported by more recent acute and chronic studies conducted
401 in healthy younger populations (Table 1). A recent meta-analysis of four randomised clinical trials
402 in older adults (55-76 y) revealed that consumption of beetroot juice did not have a significant
403 effect on blood pressure. However, consumption of beetroot juice containing 9.6 mmol/d for 3 days
404⁽¹¹⁶⁾, or 4.8-6.4 mmol nitrate/L for three weeks⁽¹¹⁷⁾ by older adults (60-70 y) significantly lowered

405 resting SBP by 5 mmHg and 7.3 mmHg respectively, compared with control. These inconsistent
406 findings highlight the need for further studies to determine effects in older population groups.

407 It was concluded from data collated from eight studies conducted in patient groups that dietary
408 nitrate may help to reduce blood pressure in hypertensive subjects, but not in patients with type 2
409 diabetes, although only one study could be found in the latter population group⁽¹¹⁸⁾. Furthermore,
410 minimal effects were reported in obese insulin resistant individuals⁽¹¹⁹⁾, and those with chronic
411 obstructive pulmonary disease despite relatively high doses of dietary nitrate (13.5 mmol/d and 9.6
412 mmol/d, respectively), although the intervention period was limited (2-3 days)^(120; 121). In contrast
413 consumption of beetroot juice (7.6 mmol/d) by 15 individuals with chronic obstructive pulmonary
414 disease significantly lowered DBP by 8.2 mmHg ($P=0.019$)⁽¹²²⁾. Additional studies are required to
415 confirm these findings.

416

417 *Platelet aggregation*

418 Dietary and supplemental nitrate have been reported to significantly reduce platelet aggregation in
419 healthy individuals^(88; 110; 123). However, a lack of effect was observed in women from one study. A
420 proposed explanation for this gender difference was reduced soluble guanylyl cyclase (sGC)
421 activity⁽¹²³⁾, a hypothesis supported by studies in mice^(124; 125), although further conformational
422 studies are required.

423

424 *Metabolic function*

425 The consumption of sodium nitrate by eNOS deficient mice reversed features of the metabolic
426 syndrome including improvements in blood pressure, bodyweight, abdominal fat accumulation,
427 circulating TAG levels and glucose homeostasis⁽¹²⁶⁾. The improvements in glucose homeostasis by
428 inorganic nitrate have been shown in a number of other mouse studies^(127; 128; 129; 130). For example,
429 Khalifi *et al.*⁽¹³⁰⁾ examined the effects of dietary nitrate in glucose tolerance and lipid profile in type
430 2 diabetic rats, and found that supplementation of drinking water with 100 mg/L sodium nitrate
431 prevented an increase in systolic blood pressure and serum glucose, improved glucose tolerance and
432 restored dyslipidaemia in an animal model of hyperglycaemia. A possible mechanism for the
433 beneficial effects of nitrate on glucose homeostasis may be the nitrite-mediated induction of
434 GLUT4 translocation⁽¹³¹⁾, which enhances cellular uptake of glucose. More recent data has also
435 shown that dietary nitrate may increase browning of white adipose tissue, which may have
436 antiobesity and antidiabetic effects⁽¹³²⁾. Yet there are few studies that have investigated the effects
437 of dietary nitrate on glucose homeostasis in humans. Gilchrist *et al.*⁽¹¹¹⁾ found that consumption of
438 250 mL beetroot juice (7.5 mmol nitrate) for two weeks by individuals with type 2 diabetes
439 increased plasma nitrate and nitrite concentrations, but did not improve insulin sensitivity measured

440 by the hyperinsulinaemic isoglycaemic clamp method. In support of this Cermark *et al.*⁽¹³³⁾ found
441 that acute ingestion of sodium nitrate (0.15 mmol nitrate per kg bodyweight) did not attenuate the
442 postprandial rise in plasma glucose or insulin following an oral glucose tolerance test in individuals
443 with type 2 diabetes.

444

445 In summary, organic nitrate is now considered to have important benefits on vascular health. While
446 these benefits include the lowering of postprandial and longer-term blood pressure in healthy
447 groups, limited data in patient groups prevents the wider translation of these findings. Nitrate-rich
448 foods have some reported benefits on measures of vascular function, with mechanistic links to
449 increasing endothelial-independent NO availability through the reduction of nitrate to nitrite, and
450 NO. The importance of the entero-salivary circulation and reduction of nitrate to nitrite by oral
451 microbiota is essential for the functional effects of dietary nitrate. Evidence for the more controlled
452 and sustained physiological effects of dietary nitrates on vascular health has prompted consideration
453 of their potential advantage over the rapid effects of nitrate supplements. Further research is
454 required to determine the lowest effective dose and specific mechanisms of action, particularly in
455 patients with hypertension and cardiometabolic disease.

456

457 **Interactions of nitrate-nitrite with flavonoids**

458 Dietary flavonoids and nitrate affect vascular health by different mechanisms. Flavonoids are
459 proposed to modulate endothelial-dependent NO release, and nitrates impact on NO production
460 from nitrite intermediate and it is possible that their combined consumption may result in additive
461 or synergistic vascular responses. Furthermore formation of NO and other reactive nitrogen species
462 in the stomach is enhanced by increasing nitrite concentrations, lower stomach pH and the presence
463 of vitamin C or polyphenols^(134; 135; 136). Bondonno *et al.*⁽¹⁰⁶⁾ investigated the independent and
464 additive effects of consumption of flavonoid-rich apples and nitrate-rich spinach. They found that
465 the combination of nitrate and flavonoids did not result in additive effects on NO status, endothelial
466 function or blood pressure, although independent effects of flavonoid-rich apples and nitrate-rich
467 spinach on these outcomes were reported. More recently, Rodriguez-Mateos *et al.*⁽¹³⁷⁾ investigated
468 interactions between cocoa flavanols and nitrate, and demonstrated additive effects on FMD
469 response when cocoa flavanols and nitrate were consumed at low doses in combination. In addition,
470 cocoa flavonoids enhanced nitrate related gastric NO formation, supporting previous studies and
471 suggests nutrient-nutrient interactions may modulate vascular function. Thus there is some evidence
472 to suggest that nitrates and flavonoids, when consumed in combination, may exert additive effects
473 on cardiovascular health, but due to the extremely limited data, confirmatory studies are required.

474

475

476

477 Conclusions

478 There is an increasing body of evidence to suggest that dietary flavonoids, particularly flavonols
479 and anthocyanidins, improve vascular function and lower blood pressure at doses achievable in
480 diets that are high in foods such as fruits, vegetables, cocoa and teas. The potential mechanisms of
481 actions are not fully understood, although increased NO availability via endothelial-dependent
482 mechanisms have been proposed as a key modulator. Cell signalling-mediated mechanisms are also
483 important in both platelet and vascular function. Dietary inorganic nitrates are also dietary
484 modulators of vascular health, primarily through the formation of NO via the nitrate-nitrite-NO
485 pathway. Promising effects of inorganic nitrate consumption on blood pressure in healthy,
486 hypertensive and other patient groups have been identified, although many of the current studies are
487 limited in power and design, particularly those in specific patient groups. It is recognised that
488 greater potential benefit may be gained from dietary nitrates compared with organic supplements,
489 with the latter causing an immediate and severe reduction in blood pressure and endothelial
490 dysfunction. Research is required to determine whether dietary nitrates can be used in combination
491 with hypotensive therapy which may reduce or eliminate the requirement for medication and the
492 associated side-effects. Consumption of diets rich in flavonoids and nitrates may be important in
493 reducing CVD risk and promoting vascular benefit, although results have been inconsistent, and
494 more long-term studies are required to determine dose-dependent effects and the specific
495 mechanisms of action.

496

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504

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Figure 1. Generic structure of a flavonoid consisting of two benzene rings linked by a 3-carbon chain

Figure 2. Structure of the seven classes of flavonoids shown as aglycones

Figure 3. Diagram of inorganic nitrate metabolism via the nitrate–nitrite–nitric oxide (NO) pathway (adapted from Hobbs et al., 2013⁽¹¹⁵⁾). A proportion of ingested nitrate (NO_3^- , \dashrightarrow) is converted directly to nitrite (NO_2^- , \rightarrow) by facultative anaerobic bacteria, that reside in plaque and on the dorsum of the tongue, during mastication in the mouth (a); the remainder is swallowed and is rapidly absorbed from the upper gastrointestinal tract. Approximately 25 % is removed from the circulation and concentrated in the salivary glands and re-secreted into the mouth, where it is reduced to nitrite. Some of the salivary nitrite enters the acidic environment of the stomach once swallowed (b), where NO is produced non-enzymically from nitrite after formation of nitrous acid (HNO_2) and then NO and other nitrogen oxides. The NO generated kills pathogenic bacteria and stimulates mucosal blood flow and mucus generation. The remaining nitrite is absorbed into the circulation; in blood vessels (c) nitrite forms vasodilatory NO after a reaction with deoxygenated Hb (deoxy-Hb). Approximately 60 % of ingested nitrate is excreted in urine within 48 h. Oxy-Hb, oxygenated Hb.

Table 1. The acute and chronic effects of dietary or inorganic nitrate on blood pressure in healthy subjects

Reference	Subject characteristics	Study design and duration	Nitrate dose and vehicle	Placebo	BP primary/secondary outcome	Effects on BP*
Jonvik <i>et al.</i> ⁽¹⁰⁷⁾	Healthy <i>n</i> =18 (11 M/ 7 F) Age: 28 ± 1 y	Acute, 0-300 min, semi-randomised, crossover	Sodium nitrate (0.58 mM/L) Beetroot juice (0.60 mM/L) Rocket salad (0.58 mM/L) Spinach (0.58 mM/L)	n/a	Primary	SBP -5 mmHg for beetroot juice (<i>P</i> <0.001), -6 mmHg for rocket salad (<i>P</i> =0.007) from 0-150 min and -7 mmHg for spinach from 0-300 min (<i>P</i> <0.001), sodium nitrate no change. DBP -3 mmHg rocket salad (<i>P</i> =0.045) and -6 mmHg spinach (<i>P</i> <0.001) from 0-300 min. No change in sodium nitrate and beetroot juice.
Ashworth <i>et al.</i> ⁽¹³⁸⁾	Healthy <i>n</i> =19 F Age: 20 ± 2 y	Chronic, 1 week, randomised, crossover	High nitrate vegetable diet	Low nitrate vegetable diet	Primary	SBP -4 mmHg (<i>P</i> <0.05) DBP no change
Jovanovski <i>et al.</i> ⁽¹³⁹⁾	Healthy <i>n</i> =27 (11 M/ 16 F) Age: 25 ± 11 y	Chronic, 1 week, randomised, placebo controlled, single blind crossover	Spinach soup (13.6 mM/d)	Asparagus soup (0.01 mM/d)	Primary	SBP -4.1 mmHg (<i>P</i> <0.01) DBP -4.4 mmHg (<i>P</i> <0.05)
Keen <i>et al.</i> ⁽¹⁴⁰⁾	Healthy <i>n</i> =6 M Age: 24 ± 1 y	Chronic, 3 days	Beetroot juice (5 mM/d)	n/a	Secondary	SBP no change DBP -12 mmHg (<i>P</i> =0.004) MAP -7 mmHg (<i>P</i> <0.001)

M, male; f, female; y, years; n/a, not available; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. * refers to differences from baseline.