

The Biological Relevance of Direct Antioxidant Effects of Polyphenols for Cardiovascular Health in Humans Is Not Established^{1–4}

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Abstract

Human studies provide evidence for beneficial effects of polyphenol-rich foods on cardiovascular health. The antioxidant activity of polyphenols potentially explains these effects, but is the antioxidant activity a reliable predictor for these effects? An International Life Sciences Institute Europe working group addressed this question and explored the potential of antioxidant claims for polyphenols in relation to cardiovascular health by using the so-called Process for the Assessment of Scientific Support for Claims on Foods project criteria. In this process, analytical aspects of polyphenols, their occurrence in foods, dietary intake, and bioavailability were reviewed. Human studies on polyphenols and cardiovascular health were reviewed together with methods for biomarkers of oxidative damage and total antioxidant capacity (TAC). In retrospective studies, F₂-isoprostanes and oxidized LDL, the most reliable biomarkers of lipid peroxidation, and measures for TAC showed the expected differences between cardiovascular disease patients and healthy controls, but prospective studies are lacking, and a causal relationship between these biomarkers and cardiovascular health could not be established. Therefore, the physiological relevance of a potential change in these biomarkers is unclear. We found limited evidence that some types of polyphenol-rich products modify these biomarkers in humans. A direct antioxidant effect of polyphenols in vivo is questionable, however, because concentrations in blood are low compared with other antioxidants and extensive metabolism following ingestion lowers their antioxidant activity. Therefore, the biological relevance of direct antioxidant effects of polyphenols for cardiovascular health could not be established. Overall, although some polyphenol-rich foods exert beneficial effects on some biomarkers of cardiovascular health, there is no evidence that this is caused by improvements in antioxidant function biomarkers (oxidative damage or antioxidant capacity). *J. Nutr.* 141: 989S–1009S, 2011.

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Introduction

Background. The putative health effects of antioxidants have attracted much attention from both consumers and the food industry, with specific interest in the large family of polyphenols. Polyphenols are antioxidants and they are ubiquitously present in plant foods, thus potentially explaining the beneficial effects of high consumption of vegetables and fruits. The common structural feature of all polyphenols, the presence of phenolic hydroxyl group(s), is the basis of their antioxidant activity in vitro and in vivo (Fig. 1) (1–3). A growing number of observational studies has examined the association between the intake of foods rich in polyphenols (onions, apples, tea, cocoa, red wine) and chronic diseases as well as the relation between the intake of individual dietary flavonoids and chronic diseases (4). Altogether, the available epidemiological evidence is most consistent for a protective effect on cardiovascular diseases (CVD).¹³ This beneficial effect is supported by a number of well-designed human intervention studies using polyphenol-rich foods that have shown consistent effects on a number of intermediate markers for CVD (5). However, isolated polyphenols have hardly been tested in human intervention studies (6).

Is there a relationship between the antioxidant activity of polyphenols and these health effects, and is the antioxidant activity of polyphenols a reliable predictor for protection against CVD? To answer this question and to explore the potential of health claims based on the antioxidant properties of polyphenols, ILSI Europe initiated a working group of experts from academia and industry. The main objective of this group was to critically review whether health claims for polyphenols based on their antioxidant activity are feasible in relation to cardiovascular health.

The guidance tools provided by the Process for the Assessment of Scientific Support for Claims on Foods (PASSCLAIM) project (7,8) were used in this evaluation. The evaluation started with a critical review of the literature regarding analysis of polyphenols, their occurrence in foods, dietary intake, and bioavailability. Subsequently, observational data as well as data from clinical trials on polyphenols and cardiovascular health were reviewed. The evidence for a causal relation between antioxidant capacity, oxidative damage, and CVD was evaluated, and methods to measure biomarkers of oxidative damage and antioxidant capacity were reviewed. Human intervention studies with polyphenol-rich foods and polyphenols that measured the effects on the most reliable biomarkers were retrieved and evaluated. Altogether, these data enabled us to evaluate whether antioxidant claims for polyphenols and cardiovascular health can be substantiated. In addition, the applicability of the PASSCLAIM criteria in this process was tested. In the present paper this evaluation is described.

The antioxidant hypothesis. Oxidative damage to biomolecules such as proteins, lipids, and DNA is thought to accumulate with ageing, leading to the pathogenesis of many age-related diseases such as, among others, CVD (9–12). In CVD, the

pathophysiological factor is atherogenesis by which plaques are formed on arterial walls that obstruct the arterial lumen. This is a chronic inflammatory process that involves a complex interplay between circulating blood components and the arterial wall (10,13,14). Oxidation of LDL is a key event in atherogenesis. Oxidized LDL (oxLDL) are efficiently taken up by macrophages resident in the subendothelial area, which subsequently form foam cells, followed by the release of cytotoxic lipid peroxidation products from oxLDL, cytokines, chemoattractant, and adhesion molecules, which all enhance the formation and growth of the plaques (14).

Oxidants in biological systems, also called reactive oxygen species, are of considerable variety, ranging from free radicals (OH^\cdot , $\text{O}_2^{\cdot-}$, NO^\cdot) to nonradicals (H_2O_2 , $^1\text{O}_2$, HOCl). They can originate from endogenous sources, such as inflammation, exercise, respiratory chain, and respiratory burst, and from exogenous sources such as cigarette smoke, pollutants, radiation, and UV light (15). Antioxidant defenses have evolved to protect biological systems against oxidants and a sophisticated cooperative array of antioxidant defense mechanisms is found in biological systems (16,17). However, despite the high grade of complexity and efficiency, the antioxidant defense system is not infallible, which has led to the concept that increased antioxidant defenses might lower the risk of diseases that involve oxidants. In particular, the direct action of supplemental antioxidants that might neutralize reactive oxygen species and thus protect DNA, proteins, and lipids from oxidative damage received much attention. This concept has been called the antioxidant hypothesis.

The antioxidant hypothesis was coined by Gey (18), who observed strong inverse associations between plasma levels of vitamin C, β -carotene, vitamin E, and selenium and ischemic heart disease mortality in a cross-cultural study. Following this hypothesis, it was thought that an increased intake of antioxidants via dietary supplements would protect against diseases where oxidative damage is involved. A substantial number of clinical trials with mainly antioxidant vitamin supplements studied the effects on CVD and cancer. However, the results of these trials were not convincing. A meta-analysis of 68 of these randomized clinical trials with β -carotene, vitamin A, vitamin E, vitamin C, and selenium supplements, either singly or combined, evaluated the effects on all-cause mortality in a total of 232,606 participants (19). Remarkably, antioxidant supplementation with β -carotene, vitamin A, and vitamin E significantly increased all-cause mortality, whereas vitamin C and selenium had no effect. Some aspects need to be considered here: the clinical trials focused only on a limited number of antioxidants, doses were higher than the RDA, and most of the supplementation studies used only 1 type of antioxidant. Besides, it is important to realize that oxidants are not only damaging but also play essential roles in a range of physiological processes. For example, the role of redox reactions in signal transduction (20) and protein structure and function (21) has become evident in recent years. The high doses of antioxidants used in the trials might have disturbed these redox processes.

As a consequence of their antioxidant activity, polyphenols might also enhance the antioxidant defense of the body. These antioxidant properties have been demonstrated in various in vitro and ex vivo models (1–3). In addition, the dietary intake of polyphenols is much higher than that of the antioxidant vitamins (“Dietary intake”). Altogether, these data suggest that supplemental dietary antioxidants might neutralize reactive oxygen species by direct action and thus protect the body from oxidative damage. The potential beneficial effects of this antioxidant

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⁴ Supplemental Tables 1 and 2 available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

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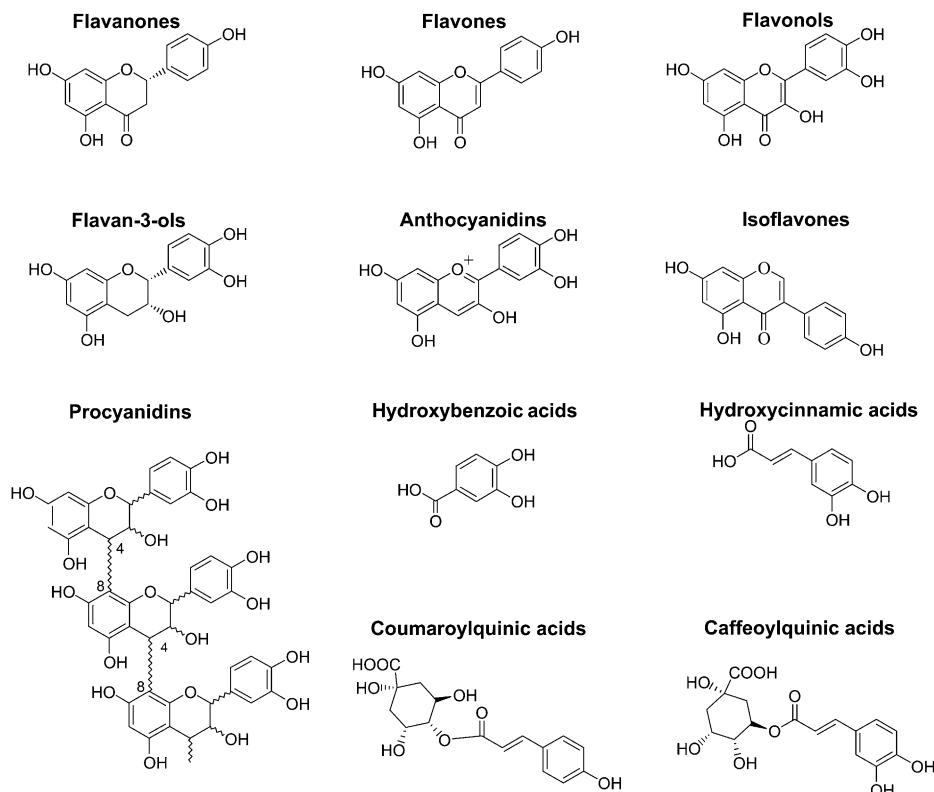


FIGURE 1 Polyphenol structures.

activity of polyphenols on CVD will be highlighted in the next sections.

Polyphenols in foods

Polyphenol structures. Several subgroups of polyphenols can be distinguished. A large subgroup is formed by flavonoids with a carbon skeleton built of 2 phenyl rings (C_6) bridged by a chain of 3 carbon atoms (C_3) forming a heterocyclic 6-membered ring with oxygen and 2 carbon atoms from an adjacent phenyl ring: C_6 - C_3 - C_6 (Fig. 1). Six subclasses of flavonoids can be distinguished: flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins, and isoflavones. A second subgroup, the phenolic acids, comprises 2 subclasses, hydroxybenzoic acids (C_6 - C_1) and cinnamic acids (C_6 - C_3) (Fig. 1). The primary structures are relatively simple, and only 9 basic hydroxybenzoic acid structures (e.g. gallic, salicylic, vanillic acid) and 4 basic cinnamic acid structures (e.g. caffeic and ferulic acid) occur in plants. Examples of other polyphenol structures that occur in plants are stilbenes (C_6 - C_2 - C_6), e.g. resveratrol, lignans (C_6 - C_3 - C_3 - C_6), and coumarins (C_6 - C_3).

Analysis of polyphenols in foods. Traditionally, methods to determine the content of all polyphenols together (total polyphenols) have been widely used in the quality management of food production and processing. These methods are based on measuring the reducing ability of all phenol groups as a sum, although not stoichiometric toward phenol groups or compounds. A common procedure is the Folin-Ciocalteu method (22) and modifications (23) that remove nonphenolic reducing compounds. Other approaches have been used that measure the total antioxidant capacity (TAC) of a polyphenol-rich food. These methods will be discussed hereafter (“Measurement of TAC of foods and body fluids”). It is obvious that these total

values do not give information on the nature of the polyphenols in the sample and are not suitable for the characterization of polyphenols in foods with a potential claimed health effect.

Many more specific analytical methods for flavonoids have been published (24). These methods cover all subclasses of flavonoids: flavonols, flavones, flavanones, flavan-3-ols (monomeric catechins, proanthocyanidins), anthocyanins, and isoflavones. Flavonoids occur in foods only as glycosylated derivatives except for the flavan-3-ols, which are present either in free (not conjugated) forms or as gallic acid esters (e.g. in tea). Glycosylation causes the very large variety of flavonoids found in plant foods, because a whole range of different sugars can be attached at various positions in the flavonoid molecule. To simplify the analytical procedure, glycosides are often hydrolyzed into their aglycones prior to detection and thus results are reported as aglycones. Analytical separation is usually performed by HPLC with reversed phase columns. Subsequently, spectral absorbance (UV/Vis), fluorometric, electrochemical detection, or MS detection systems are used for quantification or identification. Quantification of glycosylated flavonoids is often difficult, because calibration standards for glycosylated flavonoids are largely lacking. For proanthocyanidins, the oligomeric flavan-3-ols, reversed phase (25) and normal phase HPLC have been used (26), but proanthocyanidins with a large degree of polymerization may still escape scrutiny. In addition, there is a lack of calibration standards for proanthocyanidins (27).

Phenolic acids in plant products have been analyzed with a wide variety of methods based on HPLC, GC, and capillary electrophoreses (28,29). Hydroxybenzoic and cinnamic acids are generally not found in free form in plants. Hydroxybenzoic acids occur as glucosides, esters with glucose or catechins (mainly gallic acid), and oxidized dimers, as in ellagic acid. Cinnamic acids are esterified to quinic acid or to the hemicelluloses in the cell wall. Esters with other organic acids (tartaric

acid) or glucose also occur but are less widespread. In addition, glucosides of hydroxycinnamic acids are found in plants. In foods such as cereals where phenolic acids are largely bound to the cell wall, phenolic acids are determined after hydrolysis of ester bonds.

Published analytical techniques for measurement of lignans in foods include HPLC with UV, electrochemical detection, or MS detection, and GC with MS detection (30). UV detection is suitable only for quantification of high lignan contents in seeds. Coulometric detection offers adequate sensitivity but is not selective enough for a wide range of foods. A disadvantage of GC-MS is that it requires time-consuming clean-up and derivatization. Most methods were optimized for only 2 lignans, whereas at least 4 lignans may be relevant to health (30); however, more recently, methods suitable for 4 or more lignans in foods have been developed (30,31).

Due to the considerable diversity of polyphenols in foods, no single method allows analyses of all in one run. Therefore, another approach that measures polyphenol profiles has been developed (32–35).

Occurrence in foods. Polyphenols are present in virtually all foods of plant origin. About 500 different polyphenols have been reported in various foods and beverages (36–38). The polyphenol profile and content is largely determined by the type of plant species. Some plant species and their derived foods can be particularly rich in a specific polyphenol or polyphenol class: coffee in phenolic acids, red fruits and juices and red wine in anthocyanin pigments, citrus fruits and juices in flavanones, soy in isoflavones, tea in monomeric flavan-3-ols, cocoa and chocolate in flavan-3-ols (proanthocyanidins as well as monomers), and onion in flavonols (39). Some foods contain a dominant phenolic compound, e.g. epigallocatechin gallate (EGCg) in tea or chlorogenic acids in coffee, whereas other foods contain a complex mixture of phenolic compounds. For example, over 20 different anthocyanins have been identified in blueberries (40).

The polyphenol content and profile of plant foods are influenced by a range of factors: plant variety or cultivar, growth conditions (climate and soil), crop management (irrigation, fertilization, pest management), state of maturity at harvest, postharvest handling, storage, and food processing. The content of a given phenolic compound in a given food can easily double from one variety to the other. Furthermore, specific polyphenols may only be present in some varieties and absent in others. This is particularly evident for colored varieties such as blood oranges, blue potatoes, or black rice, which all contain anthocyanins that are absent in their noncolored varieties. It is therefore not surprising that the polyphenol content and profile of a given food may vary widely. Thus, representative average content values of foods can be obtained only by sampling of representative samples of different varieties collected in different regions and years.

Polyphenol content values are scattered in many scientific publications. Several food composition tables have been produced for specific polyphenol classes or subclasses by systematic analyses of representative food samples: flavonols and flavones, flavan-3-ols (catechins, proanthocyanidins), flavanones, isoflavones, and lignans (Table 1). The most comprehensive food database has been produced by the USDA and is based on a compilation of literature sources (38). It includes content values for 48 different flavonoids expressed as aglycones, meaning that individual glycosides and esters were not determined separately but were summed as aglycones after hydrolysis. More recently,

Institut National de la Recherche Agronomique in France (INRA) has developed a new Web-based database called Phenol-Explorer, a compilation of the scientific literature of 502 polyphenols so far described in foods (41). Not only flavonoids, but also phenolic acids, lignans, and stilbenes, with individual glycosides and esters are included.

The richest polyphenol food sources are shown in Table 2. Food ingredients such as herbs or spices can be very rich in flavones (e.g. sage, rosemary, thyme, peppermint), flavonols (capers, saffron), hydroxycinnamic acids (e.g. sage, rosemary, thyme, peppermint, spearmint, oregano), curcumin (turmeric), and carnolic acid (rosemary). However, due to the low quantities consumed, the contribution to total intake is generally low. In contrast, the contents of some polyphenols can be relatively low in some staple foods (e.g. chlorogenic acid in potato or quercetin in tea), but their high consumption may result in an important contribution to habitual intake.

Other polyphenols like stilbenes, chalcones, and dihydrochalcones, or dihydroflavonols, do not appear in Table 2, because there are no food sources with contents > 20 mg/100 g. In addition, although present in some foods or beverages (resveratrol in wine and nuts, phloretin in apple, etc.), their dietary intake is low or very low.

Dietary intake. Polyphenols form one of the major classes of dietary antioxidants. Their total intake is estimated to be ~900 mg/d (42). This level of intake is ~10 times higher than that of vitamin C and 100 times higher than that of vitamin E or β -carotene (43). However, several factors make the precise estimation of polyphenol intake difficult: 1) Polyphenols show a considerable diversity of chemical structures. 2) Polyphenols are present in a large variety of foods. Some polyphenols such as quercetin can be present in many foods, whereas others are often specific for a given plant species or plant family, e.g. flavanones in citrus. 3) Their content in a given food can vary to a wide extent, as a result of several factors (“Occurrence in foods”). 4) There are no standardized methods to estimate polyphenols in foods and methods of analysis can vary between publications.

For an evaluation of the health effects of dietary polyphenols, it is important to separately specify the intake of the various polyphenols, because structural changes may result in differences in biological properties (2). In addition, glycosylation of flavonoids and esterification of phenolic acids should be considered, because these modifications will affect their absorption from the gut and their bioavailability (44,45).

Today, we do not have detailed intake values for all polyphenols. Data published so far largely concern intakes of flavonoids and lignans as aglycones. Flavonoid intake data up to 2005 were reviewed earlier (46) and are updated here (Supplemental Table 1). Altogether, catechins and proanthocyanidins are by far the most abundant flavonoids in the diet, accounting for about three-quarters of the total flavonoids ingested. In coffee consumers, phenolic acids constitute a major part of the polyphenols ingested with the diet. It is evident that estimates of total polyphenol intake depend on the number of polyphenol classes or subclasses included in the survey and particularly on the inclusion of proanthocyanidins and phenolic acids. Few authors have included these 2 last groups of polyphenols.

Various factors may influence the polyphenol intake in a population. Cultural habits (e.g. soy consumption in Asian countries) and food preferences (e.g. coffee, berries) are the major factors that affect the type and amount of polyphenols ingested. Polyphenol intake has been associated with age, sex, and ethnicity, all factors known to affect food choices (47).

TABLE 1 Food composition tables and databases for polyphenols

Polyphenol class or subclass	Expression of results	Compounds, <i>n</i>	Foods	Foods, <i>n</i>	Reference
Original analyses					
Flavonols, flavones	Aglycones ¹	5	Fruits, vegetables	37	(234)
Flavonols	Aglycones	3	Beverages	12	(235)
Flavan-3-ols	Catechins	6	Fruits, beans, chocolate	36	(236)
Flavan-3-ols	Catechins	6	Beverages	10	(237)
Flavan-3-ols,	Catechins, dimers, trimers	15	Fruits, berries, vegetables, pulses, beverages	44	(238)
Flavanols-3-ols	Catechins, oligomers, polymers	6	Fruits, cereals, beans, beverages, snacks, spices	36	(239)
Flavonols, flavones, flavan-3-ols, flavanones, anthocyanins	Aglycones, catechins	20	Fruits, vegetables, nuts	51	(240)
Anthocyanins	Aglycones	6	Fruits, vegetables, nuts	24	(241)
Phenolic acids	Esters, glucosides	20	Fruits	18	(242)
Lignans	Aglycones	2	Oil seeds, cereals, fruits, berries, and vegetables	180	(243)
Lignans	Aglycones	4	Oil seeds, nuts, grain products, vegetables, fruits, beverages	109	(244)
Lignans	Aglycones	6	Various Japanese foods	86	(31)
Compilation of literature sources					
Isoflavones	Aglycones	5	Soy foods, beans	48	(245)
Isoflavones	Aglycones	6	Soy foods, beans, meat products	128	(38)
Flavonols, flavones, flavan-3-ols, flavanones, anthocyanins	Aglycones, catechins	26	Fruits, vegetables, beverages, cereals, beans, snacks, nuts, spices	231	(38)
Flavan-3-ols	Catechins, oligomers, polymers	6	Fruits, vegetables, beverages, cereals, beans, snacks, nuts, spices, baby foods	135	(37)
Isoflavones and lignans	Aglycones (isoflavones, lignans)	7	Various foods	791	(246)
Isoflavones	Aglycones	3	Korean foods	142	(247)
Phenolic acids	Free acids ²	12	Fruits, vegetables, beverages	71	(248)
Polyphenols	Glycosides and esters, catechins, flavan-3-ol oligomers and polymers	502	All foods of plant origin	452	(36)

¹ Aglycones: sum of glycosides after hydrolysis, expressed as aglycones.

² Free acids: sum of esters and glucosides after hydrolysis, expressed as free acid.

There are still gaps in our knowledge on polyphenol intakes in populations, because food composition data on some major polyphenols are still incomplete. For proanthocyanidins, there is a lack of accurate methods for their determination in foods (see “Analysis in foods”). Phenolic acids have not been comprehensively determined in foods yet. Specific foods, such as foods containing anthocyanin-based colorant additives and exotic fruits, should be analyzed. More attention should also be paid to the intake of polyphenol-containing dietary supplements, which are increasingly consumed (48).

To overcome these problems caused by missing data of polyphenols in foods, it has been suggested to use plasma or urinary levels of polyphenols or their metabolites as biomarkers of intake (49–51). However, this important field of research is still developing.

Bioavailability. The area under the plasma concentration-time curve of a polyphenol describes the fate of a polyphenol in plasma as a function of time and reflects its bioavailability. Absorption, tissue distribution, metabolism, and urinary as well as biliary excretion are separate physiological processes that all contribute to the time-dependent plasma values and determine bioavailability.

In a review, the bioavailability of a range of polyphenols as pure compounds or in polyphenol-rich products was compared after standardization to a single 50-mg dose (as aglycones) (52).

The bioavailability of flavonoids relative to that of genistein glucoside, the flavonoid with the highest bioavailability (relative bioavailability = 100%), showed large variations: isoflavones, 33–100%; flavonols, 12–41%; flavanones, 11–16%; and monomeric flavan-3-ols, 2–8%. Although no area under the plasma concentration-time curve data for anthocyanins were presented, data on their urinary excretion suggest that their bioavailability is very low.

The very low bioavailability of anthocyanins, their rapid appearance in plasma after ingestion (peak plasma values after 1.5 h), and the presence of intact anthocyanidin glycosides in the circulation all point to absorption from the stomach (53). Anthocyanidins are the only flavonoids that are absorbed from the stomach, and only anthocyanidins occur as glycosides in plasma.

The most efficient absorption, compared with stomach or colon, occurs from the small intestine because of its large surface area. Absorption from the small intestine results into peak plasma values within 1–3 h after ingestion.

Monomeric flavan-3-ols are absorbed from the small intestine. The relative bioavailability of catechin and epicatechin compared with that of genistein glucoside is 5% and that of EGCg is 2%. This rather low bioavailability of EGCg may be caused by efflux pumps associated with multidrug resistance, which regulate cellular levels (54). Especially galloylated cate-

TABLE 2 Foods and beverages richest in the different classes of polyphenols. Concentrations are extracted from the Phenol-Explorer database (36)

		Concentration range, mg/kg fresh weight or mg/L				
		200–500	500–1000	1000–5000	5000–10000	10000–15000
Anthocyanins	Wine (red)		Strawberry		Chokeberry	Elderberry
	Plum		Grape (red)	Blueberry		
	Redcurrant		Raspberry	Blackberry	Blackcurrant	
	Bean (black)		Cranberry	Cherry		
Flavan-3-ols (monomers)	Wine (white)		Olive (black)	Chocolate, cocoa		
	Wine (red)		Tea (black)	Tea (green)		
Flavan-3-ols (proanthocyanidins)	Wine (red)		Gooseberry	Wine	Chokeberry	Chocolate (dark)
				Blueberry		
				Cranberry		
	Elderberry		Redcurrant	Blackcurrant		
	Raspberry		Grape	Strawberry		
	Blackberry		Walnut	Plum		
				Apple		
				Hazelnut		
				Pecan		
				Almond		
Flavanones	Grapefruit (juice)					
	Orange (juice)					
Flavones	Olive		Artichoke (head)			
Flavonols			Chicory (red)			
	Elderberry		Chokeberry	Spinach		
	Blueberry					
	Cranberry					
	Chicory (red)					
	Endive					
	Asparagus		Onion			
	Olive					
	Beans					
	Buckwheat (wholegrain flour)					
Beans						
Isoflavonoids		Soybean				
Hydroxybenzoic acids		Cranberry	Raspberry			
	Chicory	Blackberry				
Hydroxycinnamic acids	Walnut	Chocolate				
	Lemon verbena (infusion)	Blueberry	Coffee			
			Blueberry			
			Chokeberry			
			Plum			
	Peach	Plum	Chicory			
	Common bean ²	Cherry	Artichoke (head)			
			Rye (wholegrain flour) ¹			
			Maize (flour) ¹			
			Flaxseed	Sesame meal, oil		
Lignans						
Other polyphenols		Wheat (wholegrain flour)	Olive			
		Rye (wholegrain flour)				
		Olive oil				

¹ Insoluble hydroxycinnamic acid esters bound to cell wall components.

² Concentrations are the sums of individual content values for all glycosides, esters, and aglycones of the various polyphenols in a given class.

chins such as EGCg bind to proteins in the gut, which may be another explanation for this low bioavailability (55). Information on proanthocyanidins is lagging behind, but it seems that only dimers are absorbed, whereas higher oligomers were not detected in plasma (56,57). However, absorption of dimers is

much less efficient than that of flavanol-3-ol monomers, as is evidenced by 6- to 50-fold lower peak plasma values of the dimers (52).

Most flavonoids, with the exception of flavan-3-ols, occur as glycosides in foods, and the type of sugar moiety determines

whether absorption in the small intestine is possible (58). Apart from aglycones, only glucosides are absorbable here. The impact of the type of glycoside on bioavailability is demonstrated by the large differences between quercetin glucoside (relative bioavailability 41%), which is absorbed from the small intestine, and quercetin rutinoside (relative bioavailability 12%), which can be absorbed only from the colon. Upon absorption in the small intestine, glucosides are hydrolyzed by lactase phloridzin hydrolase located at the brush border membrane (59). Thus, glucosides will not appear in the circulation.

Flavonoids that are not absorbed from the small intestine or stomach are transported to the colon, where they are subjected to metabolism by microbiota. Glycosides are hydrolyzed, which enables absorption, and peak plasma values are reached only after 4–6 h, typical for colonic absorption. Examples are quercetin rutinoside, hesperetin rutinoside, and naringenin rutinoside (52). In addition, flavonoids are broken down to a range of smaller molecules, the phenolic acids (60). As a result, the bioavailability of flavonoids that are absorbed from the colon is mostly much less than that of flavonoids absorbed from the small intestine. The notable exceptions are isoflavones that are absorbed from the colon [peak plasma values after 4.1–6.0 h (52)] but have the highest bioavailability of all flavonoids. This might indicate that isoflavones are rather resistant to degradation by microorganisms of the colon.

Phenolic acids are quite well absorbed from the small intestine. Esters of caffeic acid, such as chlorogenic acid, have a markedly lower absorption (61). Chlorogenic acid may also be absorbed from the stomach (62). Cereals are a major source of ferulic acid, which is present in esterified form to cell wall components, which hampers the absorption (63).

Most studies on lignans focused on the colonic conversion of lignans to the enterolignans enterodiols and enterolactone. Levels of enterolignans in humans are much lower than those of flavonoids and range from <0.1 to 180 nmol/L (64,65). However, in vegetarians, higher concentrations of up to 1000 nmol/L were measured (66).

Trans-resveratrol is quickly absorbed (peak plasma value after 0.5 h) (67,68). It was shown that its bioavailability was much higher than that of catechin and the peak plasma value was 4-fold that of catechin after an equal dose (67).

Upon absorption, polyphenols are readily metabolized in intestinal cells to form glucuronide and sulfate conjugates that appear in the portal blood (69). In addition, methylation of catechol units (2 phenolic OH-groups in ortho position) also may occur (46). As a result, generally only conjugated forms of polyphenols are present in blood. However, unconjugated forms of anthocyanins, EGCg, proanthocyanidin dimers (70), and some phenolic acids (60) may be present in the circulation. In the liver, additional conjugation and methylation may take place (71). It is important to realize that conjugation and methylation profoundly change the biological activity of polyphenols, a fact that has not been addressed in most *in vitro* studies, which mostly have been performed only with aglycones. For example, the antioxidant activity of quercetin conjugates is, on average, about one-half that of the aglycone (71).

The elimination half-life of polyphenols is between 1 and 18 h; however, for most polyphenols it is <8 h (52). This implies that polyphenols are mostly excreted from the body within a day after ingestion. Thus, plasma and tissue polyphenol concentrations are very dependent on the consumption pattern, and plasma values will vary substantially throughout the day. The timing of blood collection in a study is critical; fasting plasma concentrations will be much lower than postprandial values.

There is no evidence of long-term stores of polyphenols in the body. Isoflavones can be found in breast tissue of premenopausal women and in prostate glands of men. These are the only available data on flavonoid concentrations in human tissues (72,73). Animal studies show that concentrations in tissues are much lower than those in plasma (74–76).

Evidence from human studies of effects of polyphenols on CVD

Epidemiology

To date, numerous prospective observational studies have examined the association between consumption of polyphenol-rich foods or individual polyphenols and CVD risk. The importance of these observational studies is that they evaluate the human health effects of long-term exposure to physiological concentrations of polyphenols from the habitual diet. However, no information on causality can be obtained from these studies.

Foods rich in polyphenols

Associations between consumption of foods rich in polyphenols (such as tea, coffee, wine, and chocolate or cocoa) and CVD have been studied many times in various populations. A meta-analysis of 10 prospective and 7 case-control studies on the effect of black tea consumption showed protection against coronary heart disease (CHD); tea consumption across the United States and continental Europe was estimated to decrease CHD risk by an average of 11% per increase of 3 cups/d (1 cup = 125 mL) (77). However, in the United Kingdom, tea was positively associated with CHD. This is probably due to confounding, because in the UK, in contrast to the other countries, tea consumption was positively associated with a less healthy lifestyle and lower social class (78). Data from 10 mainly prospective studies on the association between green and black tea and stroke were evaluated in a meta-analysis and showed that consumption of 3 or more cups of tea (green or black) per day lowered the risk of stroke by 21% (79).

Prospective studies on filtered coffee do not provide a clear picture on the role of coffee intake and hypertension, but the risk of hypertension may be lowered in female coffee drinkers (>4–6 cups/d) (80). A meta-analysis of 21 prospective studies on the effects of filtered coffee consumption on CHD showed that moderate coffee consumption (1–4 cups/d) was associated with a decreased CHD risk only in women, whereas heavy or very heavy coffee consumption (>6 cups/d) had no effect (81).

The prospects for wine are more promising, because a meta-analysis of 10 studies (prospective and case-control) suggested that moderate wine consumption lowered CVD risk (82).

The observation that Kuna Indians consume large amounts of cocoa daily and that their mortality due to cardiovascular events is markedly low (83) fueled interest in the cardio-protective effects of cocoa. Indeed, a high cocoa intake lowered cardiovascular mortality by 50% in a Dutch cohort (84) and by 40% in a German cohort (85), but had no effect in a large U.S. study (86).

When interpreting these data, one should realize that tea, wine, coffee, and cocoa contain many other compounds than polyphenols. It is obvious that these epidemiological studies that used only food intake as a measure of exposure cannot attribute these associations to polyphenols. For instance, moderate alcohol consumption is another very likely candidate to explain the wine effect.

Individual flavonoids

Reliable data on the polyphenol contents of foods are needed to study the potential role of individual polyphenols in CVD prevention. As described in "Occurrence in foods," comprehensive data are available only for the flavonoids, with the exception of the oligomeric flavan-3-ols. Prospective cohort studies on CVD risk have evaluated the following subclasses of flavonoids.

Flavonols and flavones. To date, 10 cohort studies from 4 countries on flavonol intake and risk of CVD, mainly CHD, have been published (Fig. 2). These studies were as follows: the Zutphen Elderly (NL), a small cohort of 805 men (87); the Rotterdam (NL), a cohort of 4800 men and women (88); the Caerphilly (UK), a cohort of 1900 men (78); the Mobile Clinic (Fin), a cohort of 5130 men and women (89); the ATBC (Fin), a cohort of 25,000 male smokers (90); the Kuopio IHD (Fin), a cohort of 1950 men (91); the Health Professionals (US), a cohort of 38,000 men (92); the Iowa Women's Health (US), a cohort of 34,490 women (86); the Women's Health (US), a cohort of 38,480 women (93); and the Nurses' Health (US), a cohort of 66,360 women (94). The reported RR ranged from 0.35 (88) to 1.60 (78). Thus, high flavonol intake was associated with a small diminution in CVD risk.

Six cohort studies from 3 countries on flavonol intake and risk of stroke have been published. These studies were as follows: the Zutphen (NL), a small cohort of 552 men (95); the Mobile Clinic (Fin), a cohort of 9130 men and women (89); the ATBC (Fin), a cohort of 26,500 male smokers (96); the Kuopio IHD (Fin), a cohort of 1950 men (91); the Iowa Women's Health (US), a cohort of 34,490 women (86); and the Women's Health (US), a cohort of 38,480 women (93). A meta-analysis of these studies revealed that a high flavonol intake decreased stroke incidence with 20% (97).

Catechins, anthocyanidins, and flavanones. Data on the other subclasses of flavonoids are limited. Three cohort studies on intake of catechins, the monomeric flavan-3-ols, and CHD risk have been published. These studies were as follows: the Zutphen Elderly (NL), a small cohort of 806 men (87); the Kuopio IHD (Fin), a cohort of 1950 men (91); and the Iowa Women's Health (US) (86), a cohort of 34,490 women. The Kuopio IHD and the Iowa Women's Health studies also studied the association of CHD with anthocyanidin intake. These 2 studies and the Mobile Clinic study (89) additionally studied flavanone intake in relation to CHD risk. RR were mostly close to 1. Because of the limited number of studies, no clear

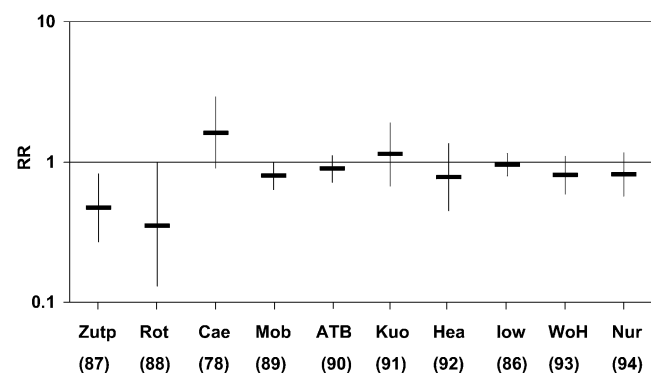


FIGURE 2 Prospective studies on flavonol (and flavone) intake and risk of CVD. RR and 95% CI are depicted. Cohorts are indicated by numbers between brackets referring to Literature Cited.

conclusions can be drawn. In the same cohorts, except the Finnish Mobile, stroke was studied. Again, the limited number of studies does not allow us to draw clear conclusions.

Isoflavones. Soy is the major source of isoflavones and most of the studies on isoflavones or soy products focused on breast and colorectal cancer. It should be borne in mind that soy is part of the habitual diet only in Asian countries. In a large Japanese cohort, an inverse association of isoflavones with CVD was found only in women (98). Mean intake of isoflavones in the highest quintile of intake was 41 mg/d. In Western countries, soy is not usually consumed, and dietary intake levels of isoflavones are <3 mg/d. An Italian case-control study (99) and 2 cohort studies in Western countries (86,100) did not find an effect of isoflavones on CVD risk. Isoflavone supplements were not considered in these studies. So, these studies confirm that it is unlikely that these low Western intake levels exert biological effects.

Lignans

Plant lignans can be converted by human intestinal bacteria into the enterolignans, enterolactone, and enterodiol, which are subsequently absorbed. It is presumed that the putative health effects of lignans are mediated by these enterolignans because of their estrogenic properties (101).

The relation between lignans and CVD was studied in 6 prospective cohort studies (Table 1). Only in 2 Finnish cohorts was a strong risk reduction of death due to CVD and CHD found. In these cohorts, serum enterodiol was measured to estimate the exposure (102,103). However, these data were not confirmed in another Finnish cohort and a Dutch cohort that also measured plasma or serum enterolignans (64,104). Two Dutch cohorts measured the intake of secoisolariciresinol and matairesinol (100), or secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol (105) and found no protective effect on CVD. Interestingly, only the intake of matairesinol was inversely associated with CHD and CVD mortality (105). Thus, effects of lignans on CVD are not convincing, because only 2 of 6 studies found a protective effect.

To summarize, epidemiological studies with polyphenol-rich foods, such as tea and wine, showed inverse associations with CVD. Observational studies with individual polyphenols mainly addressed flavonoids, and within this class most of the studies were performed with flavonols. These studies indicated a small reduction of CVD risk. The limited number of studies with other polyphenols does not allow us to draw conclusions about effects on CVD.

Randomized intervention studies and CVD risk factors

The substantiation of a health claim of a food or food component should be based on data from human intervention studies. The generally accepted standard for an intervention study is a randomized, placebo-controlled clinical trial (7,106). Almost 600 human intervention studies varying widely in the foods or extracts fed, duration, number of participants, endpoints, and quality aspects have been performed in connection with polyphenols (5). The randomized clinical trials were subjected to a meta-analysis to combine all available data on different flavonoid subclasses in relation to risk factors for CVD, e.g. blood pressure and lipoproteins, and a functional biomarker of endothelial function, flow-mediated dilatation (FMD) (5). One hundred and thirty-three trials published until June 2007 were included in the meta-analysis. Studies were included only if a suitable control arm was used as placebo so that any observed

effects could be reasonably ascribed to the intervention. This systematic meta-analysis is the most recent one and provided a snapshot of the current state of the art in relation to the relative effectiveness of the different flavonoid subclasses and highlights areas in which limited data exist.

The meta-analysis revealed that there were no randomized controlled trials that studied effects on CVD morbidity or mortality. Differential effects between flavonoid subclasses and foods on risk factors studied were confirmed by significant heterogeneity. Chocolate or cocoa increased FMD acutely [3.99% (95% CI = 2.86, 5.12); 6 studies] and chronically [1.45% (95% CI = 0.62, 2.28); 2 studies] and lowered both systolic blood pressure [−5.88 mm Hg (95% CI = −9.55, −2.21); 5 studies] and diastolic blood pressure [−3.30 mm Hg (95% CI = −5.77, −0.83); 4 studies] following chronic intake. Only soy protein isolate, but not whole soy or soy extracts, lowered diastolic blood pressure [−1.99 mm Hg (95% CI = −2.86, −1.12); 9 studies] and LDL cholesterol [−0.19 mmol/L (95% CI = −0.24, −0.14); 39 studies]. Acute black tea consumption increased systolic [5.69 mm Hg (95% CI = 1.52, 9.86); 4 studies] and diastolic [2.56 mm Hg (95% CI = 1.03, 4.10); 4 studies] blood pressure. Chronic intake of green tea lowered LDL cholesterol [−0.23 mmol/L (95% CI = −0.34, −0.12); 4 studies]. There was a greater range of data across flavonoid subclasses for TG concentrations, with evidence of a small but significant reduction following only a soy protein isolate intervention [−0.06 mmol/L (95% CI = −0.10, −0.02); 43 studies] but not with isoflavone extracts [0.01 mmol/L (95% CI = −0.05, 0.07); 14 studies]. Limited evidence is available to support an effect of red wine, chocolate, and green and black tea on TG concentrations. Individual flavonoids were inadequately studied, so there was insufficient evidence to draw conclusions about their efficacy.

The changes in risk factors and functional markers observed in the trials following intake of flavonoid-rich products are clinically important. Fifty grams of dark chocolate daily compared with placebo increased FMD by 4% acutely and 1.4% when consumed chronically. In persons at low risk of CHD, a 1.4% higher FMD response was related to a 1% lower Framingham risk score, a measure of 10-y risk of CHD (107). Chocolate or cocoa also lowered systolic blood pressure by 5.9 mm Hg following chronic intake. A decrease of 3 mm Hg would be expected to lower stroke risk by 8% and CHD mortality by 5% (108). Both green tea and soy protein isolate lowered LDL cholesterol by ~0.2 mmol/L. There is evidence from intervention studies that this level of LDL cholesterol reduction would result in a 6% reduction in both CHD-related mortality and total CHD events (109). These are profound effects, however; 50 g of chocolate (the doses typically used in the reported trials were in the range of 50–100 g/d) will provide ~15 g of fat and ~230 kcal (970 kJ), 25% of the recommended fat intake and over 10% of a mean energy intake. If this is in addition to the diet, it may not be without consequence to weight and overall dietary quality, which may counteract the observed benefits on CVD risk. To achieve the beneficial effect on LDL cholesterol, 2–5 mugs (1 mug = 250 mL) of green tea daily (up to one-half of the usual fluid intake) or 20–56 g of isolated soy protein powder, up to 75% of usual protein intake from meat or beans, would be required. Hence, the side effects and achievability of dietary regimes for reducing CVD risk need to be carefully considered.

A comparison of the effects of traditional soy foods, soy protein isolate, and isoflavone extracts suggests differences in effectiveness depending on source; only soy protein isolates seemed to be effective. For cocoa, 1 study supported epicatechin as the link between cocoa and the reported vascular effects

(110), but subgrouping by epicatechin dose (3 included studies) in the meta-analysis did not add further support for an epicatechin dose effect.

To summarize, data from over 130 randomized clinical trials provide evidence for the effectiveness of some foods rich in flavonoids or polyphenols in reduction of CVD risk factors, but it is still not clear whether these beneficial effects can really be ascribed to the polyphenols contained in these foods or extracts (6). Intervention studies with isolated polyphenols are needed. To date, only a few have been carried out to study the effects on intermediate CVD outcomes, mainly endothelial function and blood pressure, of quercetin, epicatechin, and EGCg (110–113)

Antioxidant activity and oxidative damage

Antioxidant defense of the body

Oxidants active in biological systems are of considerable variety, ranging from the high reactive hydroxyl radical to less reactive oxygen and nitrogen species with an appreciable lifetime and thus the ability to diffuse from their initial site of origin. This variety of oxidants is matched by a similar variety of antioxidant systems: the antioxidant network consisting of enzymes and low molecular weight antioxidants (16,114).

The major part of the antioxidant defense is provided by powerful enzymatic systems. These can be subdivided into those enzymes that directly interact with oxidants and those that indirectly contribute to counteract oxidation at target sites. The former include enzymes such as catalase, glutathione peroxidase/reductase, and superoxide dismutase. The latter comprise a number of enzymes that diminish levels of potential oxidants, e.g. quinone reductases to prevent potentially dangerous radical reactions. However, quinone reductases may also contribute to oxidative load by redox cycling. In addition, ancillary antioxidant enzymes perform reactions of a chemically varying nature. These include transport systems, metabolizing and conjugating enzymes (so called Phase I and Phase II), support enzymes for regenerating antioxidant molecules, metal-binding proteins, etc.

Some of the low molecular weight antioxidants are highly reactive and operative near diffusion-control, such as certain carotenoids in their reaction with singlet molecular oxygen, whereas others exert limited reactivity. Furthermore, the low molecular weight antioxidants vary from extremely lipophilic, such as β -carotene and α -tocopherol, to highly hydrophilic, such as ascorbate. α -Tocopherol can be regenerated from its oxidized form by reduction with ascorbate *in vitro*, but whether this mechanism is actively operative *in vivo* is still a matter of debate. In addition to these antioxidant (pro)vitamins, many molecules have been identified to exert antioxidant properties. Glutathione, synthesized from glutamate, cysteine, and glycine, occurs in millimolar concentration in cells but in only low amounts in plasma, where thiols play a major role. Bilirubin, the end product of heme catabolism, has also been shown to act as an efficient antioxidant. Transferrin and lactoferrin, caeruloplasmin and albumin can scavenge radicals and bind metal ions. Urate, the end product of purine metabolism from xanthine oxidase activity, is an antioxidant widely present in all body fluids and cells. Many more molecules have been identified to exert antioxidant properties, including selenium-containing compounds and secondary plant metabolites such as polyphenols (115).

The enzymes as well as the low molecular weight antioxidants are not linked via steady-state equilibria, even though

there are some redox networks. They are not uniformly distributed. Location is very stringently controlled; distinct patterns of these agents exist between different cells and organs, and, importantly, within cells (115). Furthermore, because enzymes are subject to turnover, transcriptional, translational, and posttranslational control of their activity comes into play as well. There are many other types of compounds that may or may not by themselves exhibit pronounced antioxidant activity. However, these molecules are capable of affecting redox-sensitive molecular switches that control gene expression for antioxidant enzymes, notably the transcription factor/regulator (Nrf2/Keap1) system (16).

Polyphenols might also indirectly enhance endogenous antioxidant defenses by inducing antioxidant enzymes (16). Also, polyphenols could function to prevent oxidation of food lipids prior to consumption, which could reduce the burden of prooxidant intake. However, evidence for these indirect antioxidant actions is still limited and is not considered in the present paper.

Measurement of TAC of foods and body fluids

Despite the complex human antioxidant defense system, global approaches to measure the total antioxidant activity have been applied to body fluids and tissues. A range of assays, based on redox reactions, is available (116,117). These assays measure the direct action of antioxidants with radicals and transition metals (metal chelating properties). They are easy to perform and measure in a single step the so-called TAC, a parameter that represents the cumulative effects of all antioxidants present in a sample (food, blood, or tissue). However, it is important to note that only antioxidant molecules are included and that the contribution of the antioxidant enzymes in body fluids is neglected. Roughly 2 types of assays are available. In hydrogen atom transfer (HAT) assays, antioxidants compete with a probe for radicals generated and inhibit or retard probe oxidation. These assays have the following components: a synthetic free radical initiator and an oxidizable molecular probe for monitoring reaction progress (fluorescence emission or UV absorbance). HAT assays monitor competitive reaction kinetics and the quantification is derived from kinetic curves. Common examples of HAT assays are total radical trapping antioxidant potential (TRAP), and oxygen radical absorbance capacity (ORAC) (Table 3). In single electron transfer (ET) assays, an oxidant extracts an electron from the antioxidant and changes color. The degree of color change is proportional to the effect or capacity of all antioxidants present. The reaction endpoint is reached when color change stops. In these assays, it is assumed that antioxidant capacity is equal to reducing capacity. Common examples of ET assays are ferric ion-reducing antioxidant power and Trolox equivalent antioxidant capacity (Table 3). The various TAC assays differ from each other in terms of substrates, reaction conditions, and quantification methods; thus, it is very difficult to compare the results of various assays.

Antioxidant capacity data of foods are often used for ranking purposes, e.g. comparing various sources of plant phenols or studying the effects of processing. However, these TAC assays when applied in foods do not address the all-important *in vivo* physiological processes such as bioavailability, metabolism, and tissue targeting of the antioxidants. Consequently, any claim about the bioactivity of a food solely based on these TAC values measured in the food is not justified.

In body fluids, TAC assesses the effectiveness of the endogenous nonenzymatic antioxidant network and is defined as moles of oxidants neutralized by 1 L of plasma. The main

contributor to TAC is uric acid (40–55%), followed by thiol groups (10–24%), ascorbic acid (8–15%), and vitamin E (<10%) (114). The limitations of TAC values of biological tissues and fluids should be emphasized (16). It is assumed that exposure of a biological system to an arbitrarily selected chemical radical generator or oxidant mimics the oxidants that occur *in vivo*. A second assumption is that *ex vivo* exposure of the plasma or tissue sample mimics *in vivo* conditions. This neglects basic principles of steady state and does not take into account that the major part of the antioxidant defense of a biological system is driven by enzymes. By performing an *ex vivo* challenge, these enzymes are taken out of their physiological context and are deprived of regenerating backup reactions.

Measurement of markers of oxidative damage in body fluids

Direct measurement of oxidative stress is difficult, because free radicals are highly reactive and therefore extremely short lived. Oxidative stress is a dynamic condition and is amplified by a continuing vicious circle of metabolic stress, tissue damage, and cell death, leading to increased free radical production and compromised defense systems that further exacerbate oxidative damage (118,119). Therefore, *in vivo* oxidative stress is evaluated by determining damaged molecules or products thereof. The search for biomarkers of oxidative damage led to a wide range of biomarkers measuring lipid peroxidation, DNA, and protein oxidation. A critical evaluation of the strengths and weaknesses of these markers judged that only a limited number were considered sufficiently validated to be used in studies with humans (120). Recently, the European Food Safety Authority (EFSA) disapproved the use of TBARS and the *ex vivo* oxidation lag time of LDL as biomarkers for lipid peroxidation (121). Because lipid peroxidation is the primary oxidation process that contributes to cardiovascular health (118,122,123), the focus here is on markers for lipid peroxidation.

A number of products of lipid peroxidation have been identified and measured predominately in biological fluids: conjugated dienes, hydroperoxides, aldehydes [mostly malondialdehyde (MDA) (124)], 4-hydroxynonenal (125), hydrocarbons such as pentane and ethane (in breath), F₂-isoprostanes (126), and oxLDL (127), although the last is not solely a product of lipid peroxidation. The most commonly used lipid peroxidation biomarkers are summarized in Table 4. The choice of the biomarker and the analytical method used have a great impact on the results obtained (12,119).

Isoprostanes comprise a prostaglandin-like family of compounds and are currently considered the reference biomarkers for lipid peroxidation (118,128,129). They are produced *in vivo* from arachidonic acid primarily, if not exclusively, through a nonenzymatic process of lipid peroxidation catalyzed by oxygen free radicals in cell membranes and LDL particles (130). Their production is recognized as a sensitive and reliable index of lipid peroxidation and several characteristics make them attractive markers for oxidative damage: they are specific oxidation products, stable, present in detectable quantities, increase due to *in vivo* oxidative stress, and their formation is modulated by antioxidants (117,131). In evaluating the effects of dietary interventions, urine is considered a better biological fluid than serum or plasma, because in urine accumulation of the isoprostanes can be measured, whereas isoprostanes in plasma have a short half-life time and levels vary widely across the day. MS is needed for reliable detection. Immunoassays have been developed to distinguish between the different isoprostane isomers (132) and immunoassay kits are commercially available.

TABLE 3 Characteristics of common TAC assays (117)¹

	Principle	Oxidant	Probe	Detection	Application	Main limitations
Assays involving HAT reactions						
Trapping antioxidant potential	Chain-breaking capacity	ROO [•]	R-PE	Fluorescence (495/575 nm)	Foods, body fluids, and tissues	Lipophilic antioxidants not included; nonphysiological probe
Oxygen radical absorbance capacity	Chain-breaking capacity	ROO [•]	B-PE or FL	Fluorescence (495/575 nm)	Foods, body fluids, and tissues	May be modified to include lipophilic antioxidants; protein interferences; nonphysiological probe
Assays involving ET reactions						
Ferric reducing antioxidant power	Reducing capacity	TPTZ-Fe ³⁺	TPTZ-Fe ³⁺	Absorbance (595 nm)	Foods, plasma	Thiols not included; nonphysiological pH (3.6)
Trolox equivalent antioxidant capacity	Reducing capacity	ABTS ^{•+}	ABTS ^{•+}	Absorbance (734 nm)	Foods, body fluids	Low sensitivity; nonphysiological probe.

¹ Abbreviations: ROO[•], peroxy radical; TPTZ, 2,4,6-tripyridyl-s-triazine; ABTS^{•+}, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate); FL, fluorescein; B-PE, B-phycoerythrin; R-PE, R-phycoerythrin.

However, these immunoassays are less reliable and results obtained should be regarded only as indices for isoprostane-like immunoreactivity (133,134). Concentrations of isoprostanes in blood and urine are in the picomolar range; thus, the precision of analytical methods for isoprostanes is crucial. Particular care has to be taken to prevent artificial oxidation and liberation during sampling, storage, and analysis (135). Although F2-isoprostanes are considered as reference biomarkers for lipid peroxidation, important issues still have to be resolved, such as compounds to be included, experimental conditions to be used in intervention studies, and standardization of results (128).

Oxidation of LDL is a key event in atherogenesis, because oxLDL are efficiently taken up by macrophages, which then form foam cells, followed by release of cytotoxic lipid peroxidation products and other molecules, which all enhance the formation and growth of the plaques (see "The antioxidant hypothesis"). Various components of LDL (apoB, lipids, cholesterol, unsaturated fatty acids) can be oxidized. In the last decade, immunoassays for quantification of circulating levels of oxLDL were developed using various monoclonal antibodies against oxidation-dependent epitopes of LDL. However, different monoclonal antibodies have been used, which might detect different components of oxLDL (136). Standardization of these assays, especially their specificity, is needed before oxLDL can be

regarded as a valuable biomarker. New developments using liquid chromatography with microelectromechanical systems (lab-on-a chip platform) will possibly open new avenues in the determination of circulation oxLDL (137). OxLDL are strongly correlated with plasma LDL and are thus a key factor determining absolute oxLDL concentrations. To allow for this, it has been proposed to use the oxLDL:LDL cholesterol ratio as a marker of oxidative damage (138,139).

Lipid hydroperoxides are the primary products formed when PUFA are oxidized. Analysis of lipid hydroperoxides is quite challenging, because they are reactive and decompose rapidly. Several chromatographic (GC, HPLC), enzymatic, and colorimetric methods have been developed with more or less feasibility, specificity, and sensitivity (140–142). The largest concentrations of plasma hydroperoxides were found in lipoproteins, particularly in LDL (124). The problematic nature of hydroperoxide analysis is illustrated by the wide range of plasma concentrations found in healthy humans, ranging from pmol/L to mmol/L. This huge variation can be largely explained by the different methods used for sample treatment, detection, and quantification (143–145).

MDA is one of the end products of lipid hydroperoxide degradation, mostly derived from oxidation of PUFA (146–148). Despite the fact that this marker is among the most commonly

TABLE 4 Characteristics of common lipid peroxidation assays (118, 128)

Method	Description	Comment	Reliability	References
F2-isoprostanes	Products of free radical-mediated oxidation of arachidonic acid, mostly in phospholipids	Very specific, "gold" standard. Technically complex requiring MS. Immunoassay kits not validated.	Fairly robust	(249, 250)
oxLDL	LDL modified through oxidation	Very specific. Key molecules in atherosclerosis. Various monoclonal antibodies used, comparison difficult. Commercial antibodies and immunoassay kits available	Little explored	(136, 157)
Lipid hydroperoxides	Peroxidation products of lipids	Very unstable. Require HPLC or GC-MS. Commercially available kits not validated. Calibration with various standards.	Usable with cautious interpretation	(251, 252)
TBA reactive substances (TBARS) and MDA	Peroxidation products of lipids, aldehydes (malonaldehyde). Detected by coupling to TBA.	Confounded by compounds of nonperoxidation origin. Only to be used in combination with HPLC.	Usable with cautious interpretation	(253, 254)

used, the value of MDA as a marker for lipid peroxidation remains controversial (149). This controversy is mainly caused by the various methods used to determine MDA. The most commonly used colorimetric method is based on a reaction of MDA with thiobarbituric acid (TBA). This method has been frequently criticized for its lack of specificity and its artifact formation: besides MDA, TBA also reacts with other aldehydes and non-aldehyde compounds. These TBA derivatives are unrelated to lipid peroxidation and may be formed during sample preparation (149–151). Specific methods have been developed involving chromatographic separation such as HPLC, GC, and capillary electrophoresis (152,153). However, although these methods are analytically superior, they are still not widely used.

In evaluating results obtained with these oxidative damage markers, it must be realized that no single parameter can give a general, integrative picture, even though F2-isoprostanes are currently considered the reference. Major oxidative damage may occur in a specific tissue, representing potentially only a small percentage of total body mass. As a consequence, changes in circulating oxidative damage biomarkers would be not easily induce a crucial limitation to assess the effects in human.

Relevance of antioxidant activity and oxidative damage for CVD

Antioxidant capacity in CVD

A range of pathologies has been studied, including myocardial infarction, which suggests that TAC levels in biological fluids are lower in patients than in healthy controls. However, for each disease, only a few reports have been published (116). In a cross-sectional study of ~3000 adults free of CVD, an inverse association between systolic and diastolic blood pressure and plasma TAC levels was recently observed. Prehypertensive adults had a 7% lower TAC value than normotensive adults (154). A cross-sectional study of 761 patients with type 2 diabetes showed that only males with CHD had lower plasma TAC values, but after adjustment for plasma glucose and TG, this difference was no longer significant (138). These data are all based on retrospective studies. Such studies might suggest a decrease of TAC plasma levels in oxidative stress-related pathologies or conditions. In a prospective study of 310 participants, those in the lowest quartile of plasma TAC had an approximate doubling in CHD risk, even after adjustment for well-established risk factors associated with CHD (138). However, more data that would support a causal relation between antioxidant capacity and CVD are not available.

Lipid peroxidation in CVD

Oxidative damage to LDL is one of the key events in atherogenesis (see “The antioxidant hypothesis”) and it is clear that in CVD oxidative damage is increased in the vascular wall (155,156). Peroxidation of lipids is one of the major contributors to this oxidative damage. LDL oxidation is an early event in atherosclerosis and oxLDL contributes to atherogenesis. oxLDL has a number of potentially proatherogenic activities, and several structurally unrelated antioxidants inhibit atherosclerosis in animals. Oxidative events in addition to LDL oxidation are considered important in vascular disease. These include the production of reactive oxygen and nitrogen species by vascular cells as well as oxidative modifications contributing to important clinical manifestations of coronary artery disease, such as endothelial dysfunction and plaque disruption. Increased oxLDL concentrations have been related to CVD in some

studies, although not always independently after adjustment of classical lipid markers (156,157). Recent reports suggested that the ratio of oxLDL:LDL would be a potential predictor for CVD (158). F2-isoprostanes were elevated in a range of CVD, such as atherosclerosis, coronary artery disease, heart failure, and coronary reperfusion (122,159). In addition, cardiovascular risk factors such as smoking, obesity, and type 2 diabetes were associated with increased levels of F2-isoprostanes (122,159). Although oxLDL are considered key players in atherogenesis, it remains to be established which oxidative events are a cause of rather than a response to atherogenesis (156).

Modulation of plasma TAC values by fruits and vegetables or polyphenols

Acute intervention studies with a wide range of vegetables and fruits have shown that many of these products (~80% of the foods) increased plasma TAC values. The increase in TAC values occurred immediately (30–60 min) after consumption of beverages such as tea and wine, whereas solid foods such as lettuce or chocolate displayed the TAC effect a little later, after 2–3 h (114,160–164). The effect was transient and after 4–5 h, values returned to baseline. Interestingly, foods that did not increase plasma TAC values, such as beer (165,166), white wine (167), and pear juice (168), generally had the lowest food TAC values and polyphenol concentrations (169,170).

For long(er)-term intervention studies (a few weeks to 1 y) with fruits and vegetables, the picture is less clear. In approximately one-half of the studies, an increase in plasma TAC was found after supplementation with fruits (171–176) or vegetables (163,174,177–179). Plasma TAC was significantly associated with the Mediterranean diet score and consumption of fruits, vegetables, and olive oil (180). Dietary TAC intake estimated by a FFQ correlated well with plasma TAC in Swedish women (181).

These results suggest that plant foods may increase plasma TAC values, but it is unclear which molecules are responsible for this effect. Studies with isolated polyphenols have not been performed. Is it feasible that polyphenols absorbed and present in the plasma play a role in this increase in plasma TAC values? An increase in plasma TAC values of ~50–200 $\mu\text{mol/L}$ has been measured after interventions with fruits and vegetables. Concentrations of polyphenols in plasma will certainly not be higher than 5 $\mu\text{mol/L}$ even on a high-fruit and vegetable diet (see “Polyphenols in foods”). This maximal concentration is much lower than that of endogenous antioxidants such as ascorbic acid (30–100 $\mu\text{mol/L}$) or uric acid (150–450 $\mu\text{mol/L}$) (182). In addition, the majority of these polyphenols will be present only as conjugates in plasma, which lowers their antioxidant activity appreciably (see “Bioavailability”). So, a direct contribution of polyphenols to plasma TAC is highly unlikely.

Fruits and vegetables contain many components in addition to polyphenols that might affect plasma TAC. It was shown (183,184) that the increase in plasma TAC after consumption of apples was mainly due to an increase in urate, a major contributor to TAC (116), and not to apple-derived polyphenols. An increase in urate is a well-known metabolic effect of fructose present in apples (183). However, in participants with type 2 diabetes, fructose loading did not modify plasma TAC (185). There is conflicting evidence on the role of uric acid in human health as either an antioxidant or a risk factor for CVD. Clinical evidence in humans associated hyperuricemia with insulin resistance and type 2 diabetes mellitus, obesity, hypertension, metabolic syndrome, and atherosclerosis (186). However, results from large clinical trials such as Framingham (187), NHANES I (188),

and the Japanese National Cardiovascular Survey (189) did not show an association between uric acid levels CVD or all-cause mortality. On the other hand, an inverse association of plasma uric acid and longevity has been shown in primate and non-primate mammalian species (190). One hypothesis states that during evolution, humans have acquired the ability to store uric acid, because its antioxidant properties may protect against degenerative diseases (191). The identification of a transporter molecule in human kidney that enables effective reabsorption of uric acid supports such a theory (192). In addition, it has been shown that uric acid stimulates the cell-mediated immune response (193).

Modulation of lipid peroxidation by foods high in polyphenols

As discussed before (“Measurement of TAC of foods and body fluids”), F₂-isoprostanes are currently regarded as the most reliable biomarkers of lipid peroxidation *in vivo*, whereas oxLDL are key players in atherosclerosis. So, we focused on human intervention trials using these 2 biomarkers of lipid peroxidation. For F₂-isoprostanes, we considered only intervention studies that used chromatographic methods with MS, because methods based on commercial immunoassays are not reliable (“Measurement of oxidative damage”). Thirty-four interventions, of which all but 2 (194,195) were placebo-controlled, analyzed the effects of various polyphenol-rich foods on F₂-isoprostanes and oxLDL (Supplemental Table 2). One-half of the studies used a parallel design, with mostly 10–20 volunteers in each group, whereas the other studies had a crossover design also with 10–20 volunteers. Participants were mostly healthy, nonsmoking individuals, whereas in 7 interventions, patients (196,197) or participants at higher risk for CVD, e.g. hypertension, were included (198–202). Smokers were included in only 5 studies (197,203–206).

Wine and grapes. The decrease in urinary F₂-isoprostanes in healthy nonsmokers after red and white wine consumption (207) in 1 study could not be confirmed in another study with healthy smokers that had more statistical power (203). However, in the latter study, only dealcoholized red wine decreased F₂-isoprostanes. Grape juice had no effect on isoprostanes in healthy participants (195) but decreased oxLDL in hemodialysis patients (196), whereas a lyophilized grape supplement lowered urinary isoprostanes in healthy women (208). A grape seed extract did not affect isoprostanes or oxLDL (198). Remarkably, in 2 of these studies, supplementation with antioxidant vitamins, vitamin C (198) or α -tocopherol (195), did not affect isoprostanes, nor did vitamin C show a synergistic effect with the grape seed extract.

Tea. Five of six studies performed did not find an effect of green or black tea on isoprostanes (197,199,200,209) or oxLDL (200). Even a relatively large crossover study in 66 smoking CHD patients, who supposedly would have a high level of oxidative stress, did not find an effect of tea on lipid peroxidation (197). Green tea decreased oxLDL in only 1 study (210).

Chocolate or cocoa. The effects of chocolate or cocoa were studied in 7 interventions. Wiswedel et al. (211) found only an acute decrease in isoprostanes after 2–4 h, but the effect disappeared after 6 h. This could not be confirmed in another acute study (2–6 h), where no effects isoprostanes were found (212). Two chronic studies in healthy nonsmokers did not find

an effect of chocolate or cocoa on isoprostanes (213,214). OxLDL decreased with all doses of a cocoa drink after 4 wk of intervention in healthy hypercholesterolemic participants (202). However, these investigators could not find an effect on oxLDL in normal, healthy participants (215). No effects of chocolate on oxLDL or isoprostanes were found in smokers (204). A blinded parallel study using tablets containing cacao flavan-3-ols found no effects on isoprostanes (216).

Soy or isoflavones. Soy protein isolate containing isoflavones decreased isoprostanes (217). The studies performed with soy extracts (218) or isolated isoflavones (201) did not find effects on isoprostanes.

Other products. Various fruit juices were tested without consistent effects on lipid peroxidation. An anthocyanin-rich juice (205) and pomegranate juice or apple juice (194) did not affect isoprostanes or oxLDL. In contrast, cherry juice, also rich in anthocyanins, decreased isoprostanes (219). In the latter study, an oxidative stress challenge (forearm ischemia reperfusion) was used.

A very large crossover study with olive oil rich in polyphenols in 200 healthy nonsmokers did not show effects on isoprostanes but found that only the highest dose decreased oxLDL (220). Another crossover study in the same group of people found opposite results: no effects on oxLDL but significant effects on isoprostanes (221). In contrast with the study of Covas et al. (220), the last one used a constant high dose of olive oil, only differing in amounts of polyphenols. Only isoprostanes appeared to be inversely related with the oleic:linoleic ratio in plasma, suggesting that polyphenols were not responsible for the effect.

A high flavonoid supplement (onions and tea) for 2 wk did not affect isoprostanes or oxLDL in healthy males and females (222). In a crossover study in healthy smokers, who had a low habitual vegetable and fruit intake, high daily consumption of vegetables (500 g mixed vegetables, via a vegetable burger) and fruits (330 mL fruit juice) had no effect on isoprostanes (206).

In a crossover design with 27 healthy hyperlipidemic participants, 73 and 36.5 g/d almonds decreased isoprostane excretion in urine (223).

Pycnogenol decreased isoprostanes in elderly males and females (224). However, these results are difficult to interpret, because the baseline level of isoprostanes was higher in the treatment group than in the placebo group.

Pure polyphenols. An acute study with pharmacological doses of quercetin, epicatechin, and EGCg did not show effects on isoprostanes (111). No effects of chronic pharmacological doses of quercetin on exercise-induced oxidative stress were found either (225), although exercise increased isoprostanes.

Altogether, a decrease in biomarkers of lipid peroxidation was found in only 12 of 34 intervention studies with polyphenol-rich foods or polyphenols. Only 1 of 6 tea studies showed an effect, whereas other polyphenol-rich products had no consistent effects. In the few studies with various isolated pure flavonoids, no effects were demonstrated. Because most of the studies were performed in healthy, nonsmoking individuals, it can be argued that oxidative damage levels are very low and that an effect of antioxidants would not be measurable. However, the few studies in smokers and patients do not show a different picture. In conclusion, there is limited evidence that polyphenol-rich products or polyphenols are able to decrease lipid peroxidation.

Substantiation of claims of antioxidant activity of polyphenols applying the PASSCLAIM criteria

Background and regulatory environment

Discussions in expert groups and workshops involving >160 experts from academia, industry, public interest groups, and regulators have led to consensus on an approach to evaluate the scientific support for health claims. In this PASSCLAIM project, a set of criteria for the scientific substantiation of health claims for foods has been developed (7). Here, these criteria will be applied to cardiovascular health claims of polyphenols based on their antioxidant activity.

Since the establishment of the PASSCLAIM criteria, the European claims regulation of foods came into force in July 2007 (1924/2006/EC). Two types of claims are distinguished: nutrition claims and health claims. EFSA is in charge of the evaluation of the scientific evidence supporting health claims. A claim “contains antioxidants,” overtly suggests a function in the body and a physiological or health benefit. Therefore, such a claim must be scientifically substantiated. Evidence of antioxidant activity based on capability of scavenging free radicals *in vitro* is not considered by EFSA as a proof for a beneficial physiological effect (121).

The claims regulation describes 2 types of health claims with different procedures for approval. The so-called functional health claims (1924/2006/EC, Article 13) referring to the role of a nutrient or other substance in growth, development, or functions of the body such as antioxidant function need to be based on generally accepted scientific evidence, such as textbook knowledge or consensus reports by authoritative bodies. The second type of claims are health claims of reduction of disease risk or claims targeted for children (1924/2006/EC, Article 14). In the evaluation of health claims by EFSA, the major criterion is that health claims need to be supported by scientific evidence based on human studies. The weighing of the scientific evidence is carried out according to published guidelines taking into account the PASSCLAIM criteria (106).

Application of the PASSCLAIM criteria

In the preceding sections, the current knowledge on the occurrence, analysis, and bioavailability of polyphenols and relations with oxidative damage, antioxidant activity, and cardiovascular health have been reviewed. Based on this, we applied the PASSCLAIM criteria (7) to evaluate whether antioxidant function claims for polyphenols in relation to cardiovascular health can be substantiated.

Criterion 1. The food constituent for which the health effect is claimed needs to be sufficiently characterized in terms of polyphenol identity and content. The polyphenol content and profile of a given food may vary widely (see “Occurrence in foods”). Because different biological effects are to be expected from different polyphenols, the exact nature of the active polyphenols needs to be identified. Therefore, total polyphenol methods (e.g. Folin-Ciocalteu) are not suitable, because they do not specify the various polyphenols (see “Analysis of polyphenols in foods”). TAC values of foods are not sufficient to characterize a food or food component that claims an antioxidant effect, because TAC assays in foods do not address absorption and metabolism in the body (“Measurement of TAC of foods and body fluids”). Consequently, TAC values of foods cannot predict the antioxidant effect of a food in the body. However, TAC assays could possibly be considered as quality

control measures in food processing to ensure consistency between different batches. Although standardized methods are not available, a variety of chromatographic methods for polyphenols has been developed that are able to characterize flavonoids, phenolic acids, stilbenes, and lignans. An exception has to be made for the oligomeric flavan-3-ols with a degree of polymerization > 2, where additional method development is needed (see “Analysis of polyphenols in foods”).

Criterion 2. Substantiation of a claim should be based on human data. In the present paper, we considered antioxidant effects of polyphenols on cardiovascular health. A substantial amount of observational data on polyphenol-rich products like tea, wine, and coffee, and CHD or CVD risk is available. The associations found, largely from prospective studies in various populations, are supportive of a beneficial effect on cardiovascular health of tea and wine, but not of coffee. Chocolate and cocoa have seldomly been studied in observational studies (see “Epidemiology”). Available prospective observational studies on flavonoid intake and CVD risk have focused on flavonols and support a beneficial effect of high flavonol intakes (see “Epidemiology”). A systematic analysis of human placebo-controlled interventions, in which >130 studies that complied with the quality criteria as described in criterion 2 were selected, has been performed (see “Randomized intervention studies”) (5). These studies, mostly using polyphenol-rich foods and studying risk factors of CVD, showed effects on FMD, blood pressure, and LDL cholesterol for some polyphenol-rich foods. Thus, a beneficial effect of polyphenol-rich foods on cardiovascular health is plausible; however, a direct relation with polyphenols could not be made yet. CVD morbidity and mortality has not been studied. The antioxidant hypothesis states that oxidative damage impairs cardiovascular health (see “The antioxidant hypothesis”). Hence, polyphenols might improve vascular health via their antioxidant activity, and biomarkers of antioxidant activity or oxidative damage might be surrogate markers for cardiovascular health. However, data on a causal relationship between antioxidant capacity (see “Antioxidant capacity in CVD”) or lipid peroxidation (see “Lipid peroxidation in CVD”) and cardiovascular health are not available. Overall, polyphenol-rich foods showed beneficial effects on some markers of cardiovascular health, but there is no evidence that this is caused by improvement of antioxidant function markers.

Criterion 3. When the true endpoint of a claimed benefit cannot be measured directly, studies should use markers. A rather limited number of placebo-controlled interventions, generally complying with the quality criteria as described in criterion 2, studied antioxidant function using plasma TAC values or oxidative damage biomarkers of lipid peroxidation, markers supposedly relevant for cardiovascular health. F2-isoprostanes and oxLDL are currently considered the best biomarkers of lipid peroxidation. Some evidence has been found that polyphenol-rich foods increased plasma TAC values, some of which were attributed to an increase in plasma urate concentration. The limited number of interventions studying F2-isoprostanes and oxLDL showed occasional but no convincing effects of polyphenol-rich products on lipid peroxidation. Furthermore, most of all interventions to date have studied only foods rich in polyphenols. A conclusion on an effect of the polyphenols contained in these foods is not possible, because comparison with equivalent polyphenol-poor foods is mostly lacking. In addition, studies with isolated polyphenols are scarce and no conclusions can be drawn. The link between these antioxidant

function markers and cardiovascular health could not be made, because there are no prospective studies to show that improvement of antioxidant function also improves cardiovascular health. Overall, antioxidant function claims for polyphenols in relation to cardiovascular health could not be substantiated.

Criterion 4. Markers should be biologically and methodologically valid. TAC as measured by TAC values (“Measurement of TAC of foods and body fluids”) has serious biological limitations because of the use of artificial radical generators or oxidants that might not mimic the oxidants *in vivo*. Second, in these assays, the enzymatic component of the body’s antioxidant defense is not accounted for. Third, the contribution of polyphenols to plasma TAC is negligible (“Modulation of plasma TAC values by fruits and vegetables or polyphenols”). In addition, these global assays do not allow to identify the diverse underlying metabolic changes and therefore are difficult to interpret. F2-isoprostanes, using MS, are currently considered the best biomarker of lipid peroxidation, whereas oxLDL are promising but little explored (see “Measurement of oxidative damage”). A causal link between antioxidant capacity as well as lipid peroxidation markers and cardiovascular health is not proven, because prospective studies are lacking (see criterion 2). Overall, the biological relevance of antioxidant function markers for cardiovascular health remains to be proven.

Criterion 5. Within a study, the target variable should change in a significant way, and this change should be biologically meaningful. Some of the interventions performed showed significant effects of polyphenol-rich foods on TAC values and F2-isoprostanes, but no consistent effects have been reported. Because prospective data on antioxidant function markers and cardiovascular health are lacking, it is not possible to link the magnitude of the changes found to a biologically meaningful effect.

Criterion 6. A claim should be scientifically substantiated by taking into account the total body of evidence. This criterion is self-evident and deals with giving an unbiased overview of all data in connection to the evidence for a claim. Here there are no specific comments to be made for polyphenols.

To summarize, there is no convincing evidence to show that antioxidant effects of polyphenols as measured by TAC values and biomarkers of oxidative damage are relevant for cardiovascular health in humans. Thus, health claims on direct antioxidant effects of polyphenols in relation to cardiovascular health could not be substantiated. The PASSCLAIM criteria used in this evaluation process were found to be adequate.

Discussion

This evaluation showed that the biological relevance of antioxidant effects of polyphenols for cardiovascular health is not established. Thus, health claims on direct antioxidant effects of polyphenols in relation to cardiovascular health could not be substantiated. The PASSCLAIM criteria proved to be valuable and useful tools in this process.

In vitro assays have clearly demonstrated the reactivity of polyphenols with radicals and their metal-chelating properties. These chemical properties are responsible for what can be classified as direct actions, and this aspect has received much attention in the past years. Attempts to measure this direct action *in vivo* have led to the development of biomarkers for lipid peroxidation and TAC assays. These markers have been

applied in human intervention studies with polyphenol-rich foods. In the present evaluation, F2-isoprostanes and oxLDL, the most reliable biomarkers of lipid peroxidation, and various measures for TAC were considered as indicators for a functional antioxidant effect *in vivo*. Although there is retrospective evidence for a change in these markers in CVD, prospective studies showing that an improvement in these markers also improves cardiovascular health are lacking. Thus, the physiological relevance of a potential change in biomarkers of lipid peroxidation and TAC levels is unclear. We found limited evidence that polyphenol-rich foods can modify these biomarkers. Indeed, a direct antioxidant effect *in vivo* is not feasible because of the pharmacokinetic properties of polyphenols. Polyphenols have a limited bioavailability and only low concentrations are present in the systemic circulation and tissues compared with other endogenous and exogenous antioxidants. In addition, the extensive metabolism of polyphenols during absorption and distribution in the body greatly diminishes their antioxidant activity. Thus, it can be concluded that polyphenols do not prevent oxidative damage by direct antioxidant actions.

Evidence from epidemiological studies and human interventions for beneficial effects of polyphenol-rich foods on the cardiovascular system is accumulating. Many human interventions have been performed with polyphenol-rich foods studying biomarkers of vascular function, and a substantial fraction of these interventions complied with the quality criteria defined by PASSCLAIM. To date, the effects of cocoa, tea, and soy have been the main focus of attention. Future studies should also focus on other commonly consumed subclasses of polyphenols (e.g. anthocyanins and flavanones), because they have been understudied to date. Sometimes polyphenol-rich foods have been compared with “equivalent” polyphenol-poor foods, e.g. dark and white chocolate, and in a number of cocoa studies on vascular function it was demonstrated that a role for flavan-3-ols is very likely (110,226). However, the evidence for a role of polyphenols is still poor and interventions with pure polyphenols or mixtures of pure polyphenols are needed to demonstrate their cardio-protective potential.

Because a direct antioxidant action of polyphenols is unlikely, what other mechanisms of polyphenols could be relevant? The exploration of other mechanisms for polyphenols should take advantage of the lessons learned in the last 2 decades. It is crucial to consider that polyphenols are present in the circulation and tissues only in nano- to low-micromolar ranges and are predominantly present as conjugated (mainly glucuronidated) metabolites that may also be methylated. In addition, the global whole body approach inspired by their putative direct antioxidant action should be replaced by a more targeted one (227,228).

Considering these constraints, the role of polyphenols in transcriptional gene regulation should be explored. An example is the activation of the Nrf2/Keap1 pathway by phytochemicals (229). Phytochemicals, including polyphenols, are processed by the body as xenobiotics, activating signaling pathways that result in increased expression of genes encoding cytoprotective genes. Nrf2 regulates enzymes involved in antioxidant functions or detoxification (e.g. thioredoxin reductase-1, glutathione peroxidases, heme-oxygenase-1, glutathione-S-transferases) via antioxidant-response elements, also called stress- or electrophile-response elements. Flavonoids can induce gene transcription of Nrf2 mediated by these response elements (230). Most efficient is to focus on biochemical reactions *in vivo* that affect several pathways and endpoints. It has been shown that flavonoids can modulate the activity of enzymes that are

involved in the metabolism of arachidonic acid in macrophages such as phospholipase A2, cyclooxygenase, and lipoxygenase (231). Inhibition of these enzymes by flavonoids lowers the production of prostaglandins, leukotrienes, and peroxy-nitrate, which are crucial mediators of inflammatory reactions.

Another interesting example of an approach to move away from the direct antioxidant action is the case of regulation of FMD by flavan-3-ols (227,228). FMD appears largely to be dependent on eNOS activity, the enzyme in the vessel wall that produces NO, an important vasoactive compound. There is *in vitro* evidence that tea polyphenols increase the activity of eNOS (232). It is also likely that cocoa flavan-3-ols increase eNOS activity (227) and additionally inhibit arginase, resulting in increased availability of arginine, the precursor of NO (233). Remarkably, the methylated metabolite of epicatechin is able to inhibit NADPH oxidase, thus increasing NO availability.

Progress in the field of cardiovascular health effects of polyphenols can be made when the one-dimensional antioxidant view on polyphenols is replaced by a view considering their multifaceted bioactivity. It should be realized that polyphenols are versatile bioactivities rather than mere antioxidants.

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