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JAAKKO MURSU

The Role of Polyphenols in Cardiovascular Diseases

Doctoral dissertation

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ABSTRACT

Diet rich in fruits and vegetables has been associated with a decreased risk of cardiovascular diseases (CVD). Polyphenols, such as flavonoids and phenolic acids, are ubiquitous in plants and thus potential protective compounds in them. Several epidemiological studies have suggested a high intake of dietary flavonoids to be associated with a decreased risk of coronary heart disease, but the studies on stroke are limited. In addition, out of tens of potentially health beneficial polyphenols, studies have concentrated on a few. The most popular hypothesis for the protective mechanism(s) is the ability of polyphenols to act as antioxidants, but the studies have resulted in inconsistent findings.

The aims of this work were to study the relation between dietary polyphenol intake and carotid atherosclerosis, and risk of CVD. In addition, our aim was to explore possible mechanisms of action by studying the effects of polyphenol supplementation on oxidative stress in humans.

In the first study, the association between flavonoid intake and atherosclerosis, assessed with the common carotid artery intima-media thickness (CCA-IMT), was explored in 1380 middle-aged men participating in the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) study. In this cross-sectional study, the high intake of flavonoids was associated with a decreased CCA-IMT.

In a consequent follow-up study, we studied the relation between flavonoid intake and the risk of ischemic stroke and CVD death in 1950 middle-aged men of the KIHD study. In this study, the high intake of flavonoids, especially flavonols, was associated with a decreased risk of ischemic stroke.

In a three week supplementation trial, we studied the effects of chocolate polyphenols on serum lipids and lipid peroxidation in 45 healthy, nonsmoking volunteers. We found that chocolate polyphenols increased the concentration of serum high-density lipoprotein (HDL) cholesterol in a dose dependent manner, but had no effect on lipid peroxidation.

We also assessed the effects of three week consumption of coffee (0, 450 or 900 ml/d), which is rich source of phenolic acids, on lipid peroxidation in 45 healthy, nonsmoking men. In this study, no effect of coffee on lipid peroxidation was observed.

In a four week double blind supplementation study, we assessed the effects of catechin rich phloem on serum lipids and lipid peroxidation in 75 nonsmoking, hyperlipidemic, but otherwise healthy men. In the study phloem decreased lipid peroxidation, measured as oxidation susceptibility of whole serum, while no changes in other the parameters were observed.

In summary, these results suggest that the high intake of dietary flavonoids may be associated with decreased carotid atherosclerosis and the risk of ischemic stroke. We found little support for the hypothesis that flavonoids and other polyphenols would decrease lipid peroxidation in humans.

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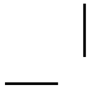
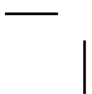
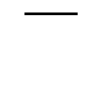
Medical Subject Headings: Antioxidants; Cacao; Coffee; Cardiovascular Diseases/prevention and control; Cerebrovascular Accident/prevention and control; Cholesterol, HDL; Diet; Double-Blind Method; Finland; Flavonoids; Fruit; Heart Diseases/prevention and control; Lipid Peroxidation; Male; Middle Aged; Oxidative Stress; Phenols; Phloem; Plant Bark; Risk Factors; Vegetables



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YHTEENVETO

Kasvisten, hedelmien ja marjojen syönnin on havaittu vähentävän riskiä sairastua sydän- ja verisuonitauteihin. Kasvikunnan tuotteiden sisältämien flavonoidien ja muiden fenolisten yhdisteiden on esitetty olevan yksi tekijä tämän suojavaikutuksen takana. Epidemiologisissa tutkimuksissa etenkin runsaan flavonoidien saannin on havaittu suojaavan sepelvaltimotaudilta. Vaikutuksia muihin sydän- ja verisuonisairauksiin, kuten aivohalvauksiin on tutkittu vain vähän. Kymmenistä päivittäin ravinnosta saatavista yhdisteistä, epidemiologiset tutkimukset ovat keskittyneet vain muutamiin. Havaitun suojavaikutuksen mekanismiksi on esitetty näiden yhdisteiden antioksidanttiominaisuuksia, vaikkakin tutkimustulokset ovat hyvin ristiriitaisia.

Tämän väitöskirjatyön tarkoituksena oli selvittää ravinnosta saatavien flavonoidien yhteyttä ateroskleroosiin sekä sydän- ja verisuonitautiriskiin keski-ikäisillä, itäsuomalaisilla miehillä Sepelvaltimotaudin vaaratekijätutkimusaineistossa (SVVT). Lisäksi selvitettiin mahdollisia mekanismeja tutkimalla flavonoidien ja fenolisten yhdisteiden vaikutuksia oksidatiiviseen stressiin, kuten veren rasva-aineiden hapettumiseen.

Ensin selvitettiin poikkileikkaustutkimuksessa ravinnosta saatavien flavonoidien yhteyttä ateroskleroosiin 1380:llä SVVT-tutkimukseen osallistuneella miehellä, joiden kaulavaltimoiden seinämien paksuutta oli mitattu ultraäänimittauksilla. Tutkimuksessa havaittiin runsaan flavonoidien saannin olevan yhteydessä merkitsevästi pienentyneeseen kaulavaltimoiden ateroskleroosiin vain vähän flavonoideja saaviin verrattuna.

Seurantatutkimuksessa selvitettiin ravinnon flavonoidien yhteyttä sydän- ja verisuonitautiriskiin 1950 SVVT-tutkimukseen osallistuneella miehellä. Tutkimuksessa havaittiin runsaan flavonoidien saannin olevan yhteydessä merkitsevästi pienentyneeseen aivohalvausriskiin vain vähän flavonoideja saaneisiin verrattuna.

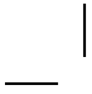
Kolmas tutkimus selvitti suklaan kaakaomassan flavonoidien vaikutuksia veren rasvoihin ja niiden hapettumiseen 45 terveellä, tupakoimattomalla tutkittavalla. Koehenkilöt nauttivat päivittäin kolmen viikon ajan 75 grammaa joko valkoista, tummaa tai kaakaon flavonoideilla rikastettua tummaa suklaata. Tutkimuksessa havaittiin tumman suklaan nostavan veren HDL-kolesterolin pitoisuutta. Kaakaon flavonoideilla ei havaittu olevan vaikutuksia veren rasva-aineiden hapettumiseen.

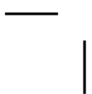
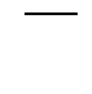
Neljäs tutkimus selvitti kahvin vaikutuksia veren rasva-aineiden hapettumiseen 45 terveellä, tupakoimattomalla miehellä. Kolme viikkoa kestäneessä kokeessa ei kahvin juonnilla havaittu olevan lyhyt- tai pitkäaikaisia vaikutuksia veren rasva-aineiden hapettumiseen.

Viides osatyö selvitti flavonoideja sisältävän, männyn kuorikerroksesta saatavan, petun vaikutuksia veren rasvoihin ja niiden hapettumiseen 75 lievästi hyperkolesterolemisella, mutta muuten terveellä ja tupakoimattomalla miehellä. Neljä viikkoa kestäneessä kokeessa havaittiin eniten pettua saaneiden miesten ryhmässä edullinen vaikutus seerumin hapettumisalttiuteen.

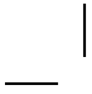
Näiden tutkimusten perusteella runsaalla flavonoidien saannilla näyttäisi olevan edullisia vaikutuksia ateroskleroosiin ja aivohalvausriskiin. Syöttökokeissa ei kuitenkaan saatu vahvistusta sille, että tutkittujen yhdisteiden suojavaikutus välittyisi niiden edullisten antioksidanttivaikutusten kautta.

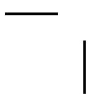
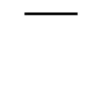
Yleinen Suomalainen Asiasanasto: antioksidantit; ateroskleroosi; aivohalvaus; flavonoidit; HDL-kolesteroli; hedelmät; Itä-Suomi; kahvi; keski-ikäiset; miehet; pettu; suklaa; polyfenolit; ruokavaliot; sydän- ja verisuonitaudit; riskitekijät; vihannekset



To my parents



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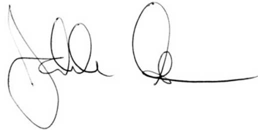


Jaakko Mursu

ABBREVIATIONS

ALAT	**A**lanine aminotransferase
AMI	**A**cute myocardial infarction
ANOVA	**A**nalysis of variance
ASAT	**A**spartate aminotransferase
ATBC	**A**lpha-Tocopherol, Beta-Carotene Cancer Prevention Study
BMI	**B**ody mass index
C	**C**arbon, e.g. in numbering C-1
CBG	**C**ytosolic β-glucosidase
CCA-IMT	**C**ommon carotid artery intima-media thickness
CHD	**C**oronary heart disease
CI	**C**onfidence interval
COMT	**C**atechol-*O*-methyl transferase
CV	**C**oefficient of variation
CVD	**C**ardiovascular disease
DC	**D**ark chocolate
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	**E**thylenediamine tetraacetic acid
ELISA	**E**nzyme-linked immunosorbent assay
FINMONICA	**F**innish part of the MONICA project
FRAP	**F**erric reducing ability of plasma
GC-MS	**G**as chromatography-mass spectrometry
GPX	**G**lutathione peroxidase
γ-GT	**G**amma-glutamyltransferase
HDL	**H**igh-density lipoprotein
HP	**H**igh polyphenol
HPC	**H**igh polyphenol chocolate
HPLC	**H**igh performance liquid chromatography
ICD	**I**nternational Classification of Diseases
IC_{50}	**C**oncentration yielding 50% inhibition
IHD	**I**schemic heart disease
KIHD	**K**uopio Ischaemic Heart Disease Risk Factor Study
LDL	**L**ow-density lipoprotein
LP	**L**ow polyphenol
LPH	**L**actase phlorizin hydrolase
MDA	**M**alondialdehyde
MI	**M**yocardial infarction
MONICA	**M**onitoring of Trends and Determinants of Cardiovascular Disease project
MUFA	**M**onounsaturated fatty acid
NO	**N**itric oxide
OHFA	**H**ydroxy fatty acid
ORAC	**O**xygen radical absorbance capacity
PBS	**P**hosphate-buffered saline
PON	**S**erum paraoxonase

PUFA	**P**olyunsaturated fatty acid
r	**P**earsons correlation coefficient
ROS	**R**eactive oxygen species
RR	**R**ate ratio
SAFA	**S**aturated fatty acid
SD	**S**tandard deviation
SOD	**S**uperoxide dismutase
TBARS	**T**hiobarbituric acid-reactive substances
tHcy	**T**otal homocysteine
TRAP	**T**otal radical trapping antioxidant parameter
UK	**U**nited Kingdom
US	**U**nited States
USDA	**U**S Department of Agriculture
UV	**U**ltraviolet
VLDL	**V**ery-low-density lipoprotein
WC	**W**hite chocolate
WHO	**W**orld Health Organization

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications referred to in the text by their Roman numerals I-V:

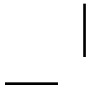
I Mursu J, Nurmi T, Tuomainen T-P, Ruusunen A, Salonen JT, Voutilainen S. The intake of flavonoids and carotid atherosclerosis: the Kuopio Ischaemic Heart Disease Risk Factor Study. British Journal of Nutrition 2007. In press.

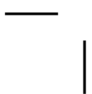
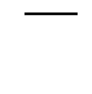
II Mursu J, Voutilainen S, Nurmi T, Tuomainen T-P, Kurl S, Salonen JT. The high intake of flavonoids is associated with decreased risk of ischemic stroke in middle-aged Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study. Submitted.

III Mursu J, Voutilainen S, Nurmi T, Rissanen TH, Virtanen JK, Kaikkonen J, Nyyssönen K, Salonen JT. Dark chocolate consumption increases HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation in healthy humans. Free Radical Biology and Medicine 2004;37:1351-1359.

IV Mursu J, Voutilainen S, Nurmi T, Alfthan G, Virtanen JK, Rissanen TH, Happonen P, Nyyssönen K, Kaikkonen J, Salonen R, Salonen JT. The effects of coffee consumption on lipid peroxidation and plasma total homocysteine concentrations: a clinical trial. Free Radical Biology and Medicine 2005;38:527-534.

V Mursu J, Voutilainen S, Nurmi T, Helleranta M, Rissanen TH, Nurmi A, Kaikkonen J, Porkkala-Sarataho E, Nyyssönen K, Virtanen JK, Salonen R, Salonen JT. Polyphenol-rich phloem enhances the resistance of total serum lipids to oxidation in men. Journal of Agricultural and Food Chemistry 2005;53:3017-3022.



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1. INTRODUCTION

Cardiovascular diseases (CVD) remain to be the main public health problem in Finland as well as in the other Western countries. Despite the decline by over 60% from the world highest mortality rates in 1960s, over 25% of all deaths in Finland are still caused by coronary heart disease (CHD) (1).

Accumulating evidence suggests that diet rich in vegetables, fruits and berries decreases the risk of CVD (2-4). Several nutritional factors in these foods, such as dietary fiber, β-carotene, vitamins C and E, are considered to contribute to the protection. Vegetables are also rich sources of flavonoids and other phenolic compounds making them candidates for the protective compounds in them. Polyphenols are a large group (>8000 compounds) of secondary plant metabolites and they are essential to the physiology of plants, with functions e.g. in growth, structure, pigmentation, and ultraviolet (UV) radiation protection (5).

Flavonoids were discovered by Hungarian scientists Rusznyak and Szent-Györgyi in 1936 who observed that some factor in a fruit juice decreased the permeability and fragility of human capillary (6). After isolating the compound from lemon juice, Szent-Györgyi named the compound "vitamin P" for permeability. Later work, however, questioned these findings showing that the results were likely attributable to traces of vitamin C in the flavonoid preparations. Further studies did not provide evidence for their vitamin status and the name was replaced with "bioflavonoids" in the 1970s. For decades, flavonoids were still considered to play a role in capillary strength, but the interest towards the effects on health waned, as no support for the absorption of flavonoids in humans was found (7).

The biological effects of polyphenols became again a focus of scientific interest in l990s. First, in 1993 Frankel et al. found that red wine polyphenols inhibited the oxidation of low density lipoprotein (LDL) *in vitro* providing a plausible explanation for the "French paradox" (7, 8). The French have low CHD mortality despite the fact that their diet is high in saturated fat, a paradox that has been suggested to be related with a high consumption of red wine (9). In 1993, Hertog and colleagues found that the high intake of flavonoids protected against CHD in the Zutphen Elderly Study (10). A few years later, Hollman et al. showed, contrary to previous findings, that flavonoids were absorbed in humans (11). These findings began a new era of polyphenol research. Several epidemiological studies have evaluated a relation between the polyphenol intake and the risk of chronic diseases such as CVD. In addition, *in vitro*, animal and human studies have assessed the mechanisms of action. The results of epidemiological studies have been inconclusive, but favoring the hypothesis that a high intake of flavonoids decreases the risk of CHD (12, 13). The most

popular hypothesis for the protective mechanism has been related with the antioxidant properties of flavonoids (14). *In vitro* and animal studies have provided strong and consistent support for this hypothesis, while the studies assessing the antioxidant effects in humans have resulted in inconsistent findings (14, 15).

The aim of this work was to study the role of polyphenols in CVD by assessing relations between the intake of polyphenols and carotid atherosclerosis, and the risk of CVD. In addition, our aim was to explore the possible protective mechanism(s) by studying the effects of polyphenol supplementation on oxidative stress, especially lipid peroxidation, in humans. In this work, the focus was on flavonoids and phenolic acids, as they are the main polyphenols in the habitual Finnish diet.

2. REVIEW OF THE LITERATURE

2.1 Cardiovascular diseases and atherosclerosis

2.1.1 Public health relevancy of cardiovascular diseases

Despite the drastic decline in CVD mortality since 1960s in Finland, in 2005 over 40% of the total mortality was still caused by CVD (1). CVD cover a collection of various heart and vascular diseases, but the main public health problems are CHD and cerebrovascular diseases. In Finland, over 25% of all deaths are caused by CHD alone, a number higher than in most Western countries. Stroke mortality is the third leading cause of total mortality causing annually over 9% of all deaths. In addition CVD include numerous other pathological events such as heart failure, hypertension, peripheral artery disease, deep venous thrombosis and pulmonary embolism, congenital heart disease, cardiomyopathy, valvular disease, rheumatic heart disease, functional disorders of the heart infections, and tumours of the heart and vessels.

2.1.2 Pathogenesis of atherosclerosis

CVD as complex diseases have multiple causes, but majority originates from the complications of atherosclerosis. The pathophysiology of atherosclerosis is caused by narrowing of the arteries resulting in inadequate blood-flow and oxygen supply for the brain, heart and/or legs. The early phase of atherosclerosis is characterized by the accumulation of cholesterol deposits in macrophages in the intima of the arteries. These foam cells can aggregate to form fatty streaks. Later, fatty streaks can mature into a fibrous plaque, which is a hallmark of established atherosclerosis. Fibrous plaque is covered by a connective tissue cap with embedded smooth muscle cells. If the plaque continues to grow, it gradually restricts the blood flow causing ischemia. The clinically important complications of atheroma usually involve rupture of a fragile fibrous cap, causing exposure of highly thrombogenic collagen and resulting intraluminal thrombus. Activated platelets trigger vasoconstriction and further propagation of the thrombus, which may result in total cease in blood flow, and subsequent clinical event (16, 17). Despite the fact that major explanatory hypotheses for the pathogenesis of atherosclerosis were proposed by pathologists von Rokitansky (18) and Virchow (19) in the mid 19th century, the pathogenesis is still not fully understood.

2.1.3 The oxidation hypothesis and atherosclerosis

Several supplemental hypotheses have been postulated to complete the theory of pathogenesis of atherosclerosis. One such is the "oxidation modification hypothesis" proposed originally in 1989 by Steinberg and colleagues (20). The basic concept of the theory is that LDL cholesterol in its native state is not atherogenic. However, the chemical modification, i.e. oxidative modification, of LDL may lead to its enhanced uptake by macrophages via "scavenger receptor pathway" resulting in foam cell formation (21). In addition, oxidized LDL is considered to be atherogenic by blocking the resident macrophages from leaving the intima, increasing the recruitment of circulating monocytes into the intima, and being cytotoxic for endothelium resulting in endothelial dysfunction (22).

Oxidative modification of LDL is initiated by "free radicals" produced in the intima by macrophages, endothelial cells and smooth muscle cells (22, 23). Free radicals are highly reactive atomic or molecular species resulting from normal oxygen metabolism in the human body (24, 25). The most common reactive oxygen species (ROS) are superoxide, hydroxyl, and nitric oxide (NO) radicals. The high reactivity of these species is related to unpaired electron or electrons. Increased production of ROS can cause oxidative damage to nucleic acids, lipids, proteins and carbohydrates. Especially double bonds of polyunsaturated fatty acids found e.g. in the LDL cholesterol are prone to oxidative modification. On the other hand, ROS also have an essential role in several beneficial physiological functions e.g. regulating the vascular tone, antimicrobial activity and regulation of cellular proliferation and growth.

Humans have effective antioxidant defence which includes antioxidant enzymes, as well as non-enzymatic radical scavengers referred to as antioxidants. The enzymes include e.g. superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). The role of antioxidant enzymes is to maintain a reducing tone within cells. The most extensively studied non-enzymatic radical scavengers are β-carotene, vitamin E, vitamin C, urate and thiols. Normally, the antioxidant defence system stay in balance with the production of oxygen-derived species. The balance may be disrupted e.g. by depletion of antioxidants or increased formation of ROS leading to "oxidative stress" (26). Increased formation of ROS is suggested to result e.g. from smoking, chronic inflammation, drugs, or toxins, while depletion of antioxidants can be caused e.g. by inadequate intake of antioxidant vitamins. According to the "antioxidant theory", dietary antioxidants, such as vitamin C and E, can decrease oxidative stress and lipid peroxidation, and thus prevent atherosclerosis (23).

2.1.4 Measurement of oxidative stress

The assessment of oxidative stress in human body has turned out to be a challenging task, and currently there is no agreement with regard to a reference method(s). Several different approaches have been used, such as different measurements of total antioxidant capacity, lipid peroxidation, and activity of antioxidant enzymes.

The methods most often used for measuring total antioxidant capacity are total radical trapping antioxidant parameter (TRAP) and oxygen radical absorbance capacity (ORAC) (27, 28). In these measurements, after induction with a pro-oxidant, the oxidation of hydrophilic fraction of plasma or serum is monitored. Another approach is to measure the ability of hydrophilic fraction of plasma to quench radicals without pro-oxidant induction. Example of such a method is ferric reducing ability of plasma (FRAP) assays.

Several methods have been developed to assess the lipid peroxidation. Measurement of thiobarbituric acid-reactive substances (TBARS) is a robust method based on measuring end products of peroxide breakdown, such as malondialdehydes (MDA) (29). *Ex vivo* oxidation susceptibility of isolated LDL fractions or whole serum are among the most widely used methods (29). In these methods, oxidizing agents such as transition metals are used as initiators of lipid peroxidation and oxidation is assessed by measuring the formation of conjugated dienes. F_2-isoprostanes are oxidation products of arachidonic acid and their levels are mainly determined by gas chromatography-mass spectrometry (GC-MS) or by enzyme-linked immunosorbent assay (ELISA) (30). Currently, F_2-isoprostanes are considered to be the most reliable marker of *in vivo* lipid peroxidation (31-34). Hydroxy fatty acids (OHFAs), which are oxidation products of unsaturated fatty acids, are also *in vivo* markers of lipid peroxidation (35). In addition, the activity of various antioxidant enzymes, such as blood/plasma GPX, SOD or catalase, are considered to reflect the state of oxidative stress in the human body.

The aforementioned methods are widely used for assessing the effects of antioxidants on oxidative stress in humans. However, the relevancy of these measurements as a predictor of atherosclerosis and CVD is unclear (34, 36).

2.1.5 Risk factors for cardiovascular diseases

A substantial number of factors are considered to contribute to the risk of CVD. The major and independent risk factors are male gender, cigarette smoking, elevated blood pressure, elevated serum total cholesterol and LDL cholesterol, low serum high-density lipoprotein (HDL) cholesterol, diabetes mellitus, and ageing (37). In addition, obesity and low physical activity are

major predisposing risk factors. Potential risk factors include e.g. elevated plasma total homocysteine (tHcy), prothrombotic factors, and inflammatory markers, but their causative, independent, and quantitative role has not been clearly established.

Numerous nutritional factors are also considered to modulate the risk. The high intake of saturated fatty acids (SAFA), salt, and alcohol are considered to increase the risk, while the high intake of mono- (MUFA) and polyunsaturated (PUFA) fatty acids, whole grains, fruits and vegetables decrease the risk (38). The protective compounds in fruits and vegetables are not entirely known, but fiber, various vitamins and other micronutrients are thought to be responsible. Vegetables are also rich in polyphenols, such as flavonoids, and these compounds may also play a role in the protection.

2.2 Polyphenols

2.2.1 Nature of polyphenols

Polyphenols are a wide and complex group of secondary plant metabolites (39-41). So far, over 8000 compounds have been identified. Structures of the compounds range from simple molecules such as phenolic acids, to highly polymerized compounds like proanthocyanidins. Polyphenols are essential for the physiology of plants, having functions in growth, structure, pigmentation, pollination, allelopathy, and resistance for pathogens, UV radiation and predators (5). In addition, sensory qualities of plant foods and beverages, such as astringency and bitterness are related to their polyphenol content (39). In nature, polyphenols occur primarily in conjugated forms with one or more sugar residues attached to hydroxyl groups (39, 41). Conjugation increases the polarity of the molecule, which is necessary for storage in plant cell vacuoles. The most common sugar residue is glucose, and residues can be in the form of monosaccharides, disaccharides or oligosaccharides. In plants, polyphenols are relatively resistant to heat, oxygen, dryness, and to some extent also to acidity, but the sensitivity to light differs according to their chemical structure (41, 42).

Out of the large variety of compounds, the most common groups of phenolic compounds are phenolic acids and flavonoids (39, 41) (**Figure 1**). Phenolic acids can be further distinguished into two groups, hydroxybenzoic acids and hydroxycinnamic acids (40). Flavonoids represent the most common and widely distributed group of polyphenols. Insofar, thousands of flavonoids have been identified (41). Flavonoids have diphenylpropane (C6-C3-C6) structure, which consists of two or more aromatic rings connected with three carbons. The basic structure of a flavonoid allows a wide

variety of different substitution in the A, B, and C rings, resulting in multiple subclasses (43). The main substituents are hydroxyl, methoxyl, or glycosyl groups, which can be further substituted forming very complex structures (44). Classification of flavonoids into subclasses is based on the functional groups in the C ring. Subclasses include anthocyanidins, flavan-3-ols, flavones, flavonols, flavanones, and isoflavonoids. This work did not include isoflavonoids because the intake of these compounds is generally low in Finland. Oligomeric or polymeric forms of flavan-3-ols are referred to as proanthocyanidins or condensed tannins (44). The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (41). In nature most flavonoids occur as glycosides (i.e. aglycone conjugated with sugar moiety), except flavan-3-ols which occur as aglycones (43).

2.2.2 Occurrence of polyphenols in foods

Polyphenols are ubiquitous in plant kingdom and practically all plant foods and beverages contain at least some amounts of these compounds (39-41). The richest sources are fruits, berries, vegetables, cereals, legumes, nuts, and beverages such as wine, tea, coffee and cocoa. However, the types and amounts of compounds may vary greatly between different foods (**Table 1**).

Out of two groups of phenolic acids, hydroxybenzoic acids and hydroxycinnamic acids, the latter is much more common (40). The most common hydroxycinnamic acids are caffeic and ferulic acid (45). Caffeic acid mainly occurs in esterified form with quinic acid forming chlorogenic acid. Caffeic acid and chlorogenic acid are present in very high amounts in coffee (46). Ferulic acid is present in food items rich in cereals (40, 47).

Flavonols occur widely in fruits and vegetables as well as in beverages (40, 45, 48). Quercetin, which is the most common flavonol, is especially rich in onions and kale (40). Flavan-3-subclass, which consists of both monomeric (catechins) and polymeric (proanthocyanidins) forms, are one of the most ubiquitous flavonoids in plant foods (40). Rich sources of flavan-3-ols are berries, nuts, dark chocolate, red wine, green and black tea. Anthocyanidins are pigments of red fruits and berries, and high amounts of anthocyanidins are found in black currants, blueberries, black grapes, cherries, and rhubarb (40). Citrus fruits and citrus juices are the main sources of flavanones. Flavones are less common, but significant amounts can be found in parsley and celery.

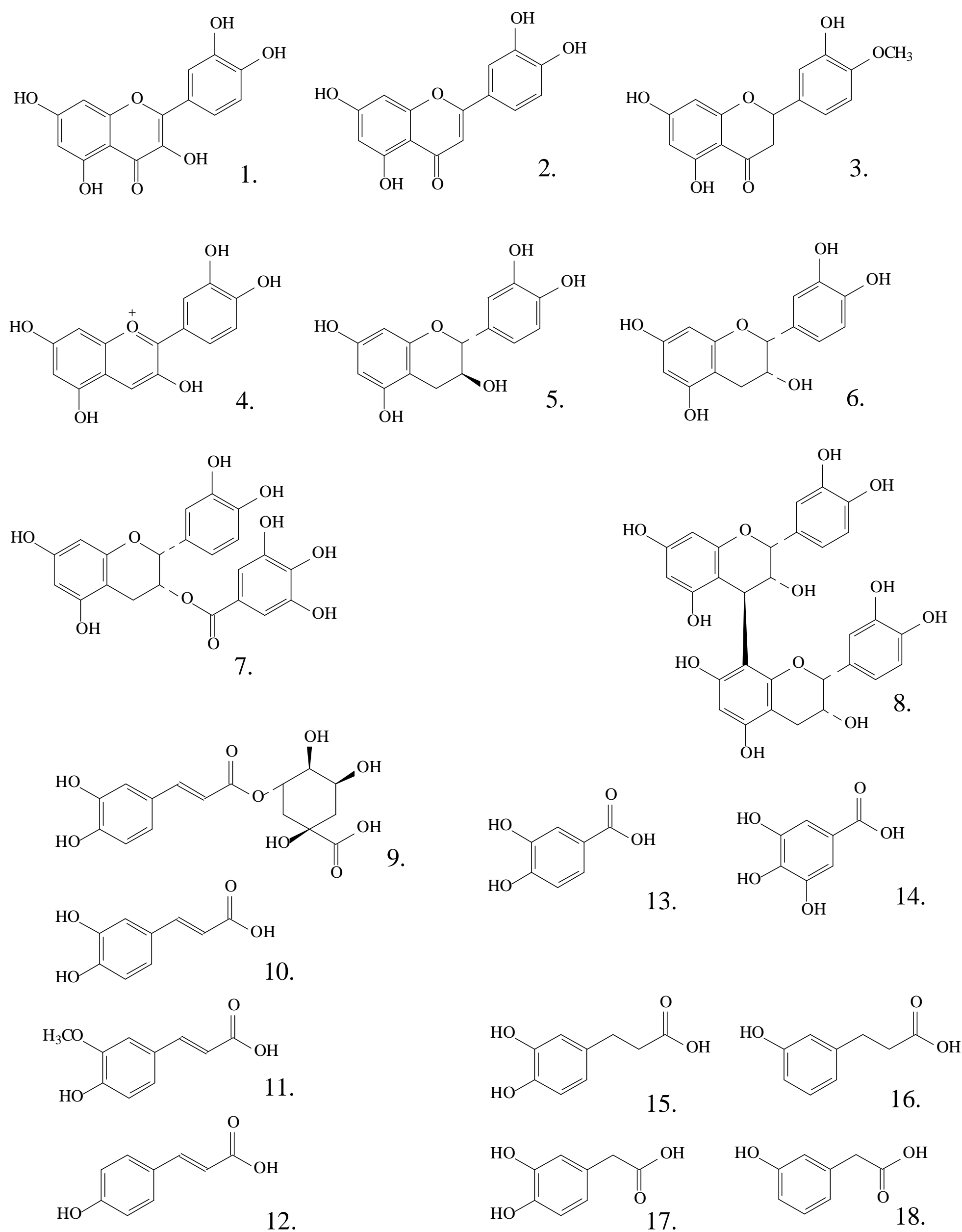

Figure 1. Structures of selected flavonoids and phenolic acids. 1. Quercetin (flavonol), 2. Luteolin (flavone), 3. Hesperetin (flavanone), 4. Cyanidin (anthocyanidin), 5. Catechin (flavan-3-ol), 6. Epicatechin (flavan-3-ol), 7. Epicatechin-3-gallate (flavan-3-ol), 8. Epicatechin-(4β-8)-epicatechin (proanthocyanidin), 9. Chlorogenic acid, 10. Caffeic acid, 11. Ferulic acid, 12. *p*-Coumaric acid (9-12, cinnamic acid derivatives), 13. Protocatechuic acid, 14. Gallic acid (13-14, benzoic acid derivatives), 15. 3,4-Dihydroxyphenylpropionic acid, 16. 3-Hydroxyphenylpropionic acid, 17. 3,4-Dihydroxyphenylacetic acid, 18. 3-Hydroxyphenylacetic acid (15-19, propionic and acetic acid derivatives, metabolites).

Table 1. Polyphenols in foods

Subclass	Individual compounds	Primary food sources	Polyphenol content (mg/100g)
Hydroxybenzoic acids	Gallic, vanillic, syringic, *p*-hydroxybenzoic acid	Black currant	4-13
		Raspberry	6-10
		Strawberry	2-9
Hydroxycinnamic acids	Caffeic, ferulic, *p*-coumaric, sinapic acid	Blueberry	100
		Coffee beverage	35-175
Anthocyanidins	Cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	Black currant	130-400
		Black grape	30-750
		Blueberries	25-500
		Cherry	35-450
		Rhubarb	200
		Strawberry	15-75
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin	Apple	2-4
		Broccoli	4-10
		Cherry tomato	1.5-20
		Kale	30-60
		Leek	3-23
		Onion	35-120
Flavones	Luteolin, apigenin	Celery	2-19
		Parsley	24-184
Flavanones	Hesperetin, naringenin, eriodictyol	Grapefruits juice	10-65
		Lemon juice	5-30
		Orange juice	20-70
Flavan-3-ol monomers	Catechin, epicatechin, gallocatechin, epigallocatechin, epicatechin-3-gallate, epigallocatechin-3-gallate, theaflavin, theaflavin gallate, theaflavin digallate, thearubigins	Apple	2-12
		Apricot	10-25
		Black tea beverage	6-50
		Cherry	5-24
		Chocolate	46-60
		Grape	3-18
		Green tea beverage	10-80
		Peach	5-14
		Red wine	8-30
Flavan-3-ol polymers (procyanthocyanidins)		Almond	184
		Apple, with peel	43-136
		Blueberry	329
		Cranberry	419
		Dark chocolate	234
		Hazelnut	501
		Plum	247
		Red wine	62
		Strawberry	142

Modified from (40, 49).

The polyphenol content in plants is mainly determined by genetic factors such as plant phyla, order, family and population variations within species (41, 48). Several environmental factors, such as light, climate, and seasonal variation also affect the types and the amounts (50). Biosynthesis of polyphenols is stimulated by sunlight and thus sunny climates usually increase the concentrations. Due to sunlight, the highest concentrations of phenolic compounds are usually found either in plant

leaves or in the skin of fruits, while only minor amounts are found in the inner parts (39, 41). Other factors such as the degree of ripeness influence the amount as well as the types of compounds found in foods (40). In general, higher the degree of ripeness, lower the amounts of phenolic acids and higher the content of flavonoids.

Polyphenol content of foods is to some extent affected by food processing and cooking (43, 44). In fruits, flavonoids are almost completely found in the peel, and thus peeling causes great losses. For example, peeling the apple eliminates almost all quercetin (51). Processing can also cause transformation of polyphenols. For example, the oxidation process in the fermentation of green tea to black tea causes reduction of flavan-3-ols with a concomitant increase in theaflavins and thearubigens (43, 48).

The knowledge of the polyphenol composition of plant foods is still incomplete. This is mainly because methods for detecting some polyphenols, e. g. anthocyanidins, from foods have been available only for a few years. In addition, there is a vast diversity of compounds and a huge amount of plant foods that needs to be analyzed. Also, the lack of agreement on the appropriate methods to analyze the content makes comparison between results from different studies very difficult. Apples are considered to be one of the few foods for which the composition has been described in detail. (40, 45)

2.2.3 Dietary intake and sources of polyphenols

The earliest estimations about the daily intake of polyphenols was proposed in 1976 by Kühnau, who suggested that the average intake in the United States (US) would be around 1 gram per day (42). This estimate was, however, presented at a time when no reliable methods to analyze polyphenol content of the foods were available. Appropriate analytical methods were developed in 1990s, but most of the more recent calculations on the intake are still suggestive as they have mainly included only two flavonoid subclasses, flavonols and flavones (**Table 2**). It has been estimated that at least five subclasses, a total of 20-30 individual flavonoids, are common in daily diet (52).

In addition to flavonoids, phenolic acids contribute significantly to the daily intake, but data for their intake is limited. Also, estimates may not be representative for the whole population. Data is mostly derived from the epidemiological studies which have assessed the relation between the intake and disease in some specific subgroup of population (e.g. elderly men), and not the absolute intake at population level.

Table 2. Population based surveys estimating mean daily flavonoid intake[1]

Study and reference	Country	Age (y), gender	Dietary method	Flavonoid subclasses	Intake (mg)	Main dietary sources
ATBC Study (53)	FI	50-69 M	Dietary history	Flavonols Flavones	9.7 0.1	Vegetables, tea, fruits and berries
Finnish Mobile Clinic Health Examination Survey (54)	FI	≥15 M+F	Dietary history	Flavonols Flavones Flavanones	4.0 <0.1 20.2	Apples, onions, white cabbage, citrus fruits, berries
Zutphen Elderly Study (10, 55)	NL	65-84 M	Dietary history	Flavonols, flavones	25.9	Tea (61%), onions (13%), apples (10%)
				Flavan-3-ols	72	Black tea (87%), apples (8%)
Rotterdam Study (56)	NL	≥55 M+F	FFQ	Flavonols	28.6	Not reported
Caerphilly Study (57)	UK	45-59 M	FFQ	Flavonols	26	Tea (>80%)
Health Professional Follow-up Study (58)	US	40-75 M	FFQ	Flavonols, Flavones	19.9 0.2	Tea (25%, onions (25%), apples (10%), and broccoli (7%)
Iowa Women's Health Study (59, 60)	US	55-69 F	FFQ	Flavonols Flavones	13.8 <0.1	Tea (36%), apples (17%), and broccoli (9%)
				Flavan-3-ols	25.4	Tea (59%), apples and peas (26%), chocolate (6%), and other fruits than apples or pears (5%)
Women's Health Study (61)	US	≥45 F	FFQ	Flavonols, Flavones	24.6	Tea (31%), onions (23%), broccoli (8%), and apples (8%)

[1]ATBC=Alpha-Tocopherol, Beta-Carotene Cancer Prevention, F=females, FFQ=food frequency questionnaire, FI=Finland, M=men, NL=Netherlands, UK=United Kingdom, US=United States.

Currently, a lot of data for the intake of flavonols and flavones in Western populations is available. The daily intake of flavonols has varied from a few to tens of milligrams, while the intake of flavones has been mainly less than 1 mg. In Finland, the intakes of flavonols and flavones are slightly less than generally in Europe and US. The intake of flavanones has rarely been studied, and the intake has been reported to be 20 mg in Finland (54) and 58 mg in Greece (62). Limited amount of calculations has been made for monomeric catechins (flavan-3-ols), and the daily intake has been 72 mg in the Netherlands (55), 25 mg in the US (60) and 11 mg in Greece (62). Only a few estimates for anthocyanidin intake are available, but as their concentrations are high in several commonly consumed foods, the daily intake could be hundreds of milligrams (40). Similarly, no reliable estimates of the intake of proanthocyanidins are available, but it has also been suggested to be hundreds of milligrams per day (63). The intake of phenolic acids is strongly dependent on coffee consumption. Coffee is rich in phenolic acids (~100 mg/dl) and thus among coffee drinkers the daily intake can easily be several hundreds of milligrams (46). In summary, reliable estimations

of intake of many polyphenols are still lacking, but the original suggestion for the total intake of polyphenols may eventually end up being surprisingly accurate.

The main sources of flavonoids have been reported to be tea, onions, fruits, especially apples, berries and broccoli with some variation between countries. However, thorough evaluation of the sources is possible when the food composition data and estimates about the intake are complete.

2.2.4 Bioavailability and metabolism

To induce biological effects *in vivo*, sufficient amount of polyphenols have to be absorbed and reach the target tissues. Bioavailability studies are an important part of the puzzle when determining the compounds which could have effects on biological functions. Due to a vast diversity of polyphenolic compounds, an extensive metabolism, and methodological difficulties in studying the metabolism in the human body, the knowledge is still limited. Studies have mainly focused on measuring concentrations in plasma and/or urine after consumption of pure compounds, plant extracts or foodstuffs rich in polyphenols (64).

Absorption of polyphenols

For decades, polyphenols were considered not to be absorbed (42). This was because in foods the majority of polyphenols occur in glycosylated forms, and enzymes capable of cleaving the glycosidic bonds were not known to be secreted in the gut. Absorption was thought to be possible only for aglycones which, however, only occur in minor amounts in foods. In the early work of Gugler and colleagues in 1975, they were not able to detect quercetin in human plasma or urine after supplementation of purified quercetin aglycone (7) and thus it was reasoned that even aglycones are not absorbed.

Later in 1995, Hollman and colleagues challenged these early findings by showing in their studies that quercetin was absorbed (11). They also found that the glucosides were absorbed more efficiently than aglycones. The absorption of quercetin glucosides from onions was found to be 52%, while absorption of pure aglycone and quercetin rutinoside were 24% and 17%, respectively. Similar findings have been later reported also by others (65, 66). Plasma concentrations of flavonols have mainly varied from 0.13 to 7.6 μmol/l after the supplementation (64). The bioavailability of flavan-3-ols has varied between compounds (64). Catechin monomers are relatively efficiently absorbed, while polymeric forms (proanthocyanidins) are poorly absorbed. Plasma concentrations of monomers have mainly been <1 μmol/l, but consumption of cocoa has yielded higher concentrations. The poor bioavailability of proanthocyanidins is thought to be related to their polymeric structure and large molecular weight. Absorption is thought to be limited

to only some dimers (B2) (40). The bioavailability of anthocyanidins is also considered to be poor. After consumption of berry products, the concentrations in plasma have only been 10-50 nmol/l (64). However, to some extent the absorption may have been underestimated because of difficulties in the analysis (64, 67). Some important metabolites may have been neglected and the poor stability of anthocyanidins in biological samples has complicated the work further. The bioavailability of flavanones and flavones is scarcely studied. Absorption of flavanones is considered to be relatively efficient and after the supplementation the plasma concentrations have varied from 1 to 6 µmol/l (64).

Bioavailability of phenolic acids have been evaluated much less than flavonoids, and studies have concentrated mostly on hydroxycinnamic acids; caffeic acid and chlorogenic acid (64). Absorption of caffeic acid is efficient, but esterification reduces the absorption significantly. Olthof et al. (68) found out that in ileostomy subjects, absorption of the caffeic acid was as high as 95%, while for chlorogenic acid the absorption was reduced to 33%. The absorption of phenolic acids is rapid and peak plasma concentrations are found at 1 h (69). Ferulic acid is efficiently absorbed from high bran cereals (70) or beer (71) when present in free form, while esterification hampers the absorption.

In general, the bioavailability of most polyphenols is relatively poor (64, 67). The rate and extent of intestinal absorption and the metabolites found in plasma and urine are largely determined by the chemical structure of the compound (39, 45, 64). The concentrations in plasma vary usually between 0 to 4 µM after polyphenol supplementation (45, 64). Absorption is quite rapid and the peak concentrations in plasma are measured 1-2 h after ingestion. The absorption is considered to be dose-dependent even though this has been demonstrated only for one compound, epigallocatechin-3-gallate (72). Even though scarcely studied, the food matrix may affect the bioavailability, and basically concomitant consumption of any food may hinder the absorption (40). Variation also exists between individuals in the absorption and metabolism of phenolic compounds which may be related e.g. to differences in the microflora or intestinal enzymes (64, 66).

Small intestine and colon are the main sites of polyphenol absorption (**Figure 2**). Some aglycones, such as quercetin, can be absorbed at the gastric level, but not glycosides which are resistant to acid hydrolysis in the stomach (73). In the small intestine, minor part of the polyphenols can be absorbed by passive diffusion (39, 40). Those compounds include free phenolic acids and flavonoid aglycones. In foods, most polyphenols are in the form of esters (glycosides or polymers) which cannot be absorbed as such, but must first be hydrolyzed to aglycones by gastrointestinal mucosa or colonic microflora (45). In the small intestine, glycosides are deglycosylated either by lactase phlorizin hydrolases (LPH) or cytosolic β-glucosidases (CBG) (74, 75). Aglycones are then

able to diffuse into the intestinal cells. Glycosides may be transported into the enterocytes without hydrolysis by sodium-dependent glucose transporter and then hydrolyzed by CBG.

Those compounds that are not absorbed enter colon and are hydrolyzed by microflora (40). The formed aglycones are then further metabolized to phenolic acid derivates which might be absorbed later. Absorption in the colon is considered to be less efficient than in the small intestine because of the smaller exchange area and lower density of transporters (64). The role of microflora in the metabolism may be more significant than currently estimated and the amount of microflora-derived metabolites in the blood as well as in the urine may be higher than tissular metabolites (40).

Metabolism of polyphenols in the body

After absorption into the mucosa, the aglycones are conjugated in the small intestine or later in the liver (40). Polyphenols are mainly either methylated, sulfated or glucuronidated. Conjugation restricts their potential toxic properties and enhances their biliary and urinary excretion. The enzyme involved in methylation is catechol-*O*-methyl transferase (COMT). COMT is found in wide variety of tissues, but the highest activity is found in the liver and kidneys. In the circulation polyphenols are primarily bound to albumin (40). The metabolism of anthocyanidins may differ from other flavonoids. For most polyphenols only traces of their native forms are found in plasma or urine, while unchanged anthocyanidin glycosides have been the main forms detected. Recently, methylated and glucuronidated forms of anthocyanidins have been detected (76-78).

A very little is known about the tissue uptake of polyphenols in humans. Polyphenols have been detected in wide range of mouse and rat tissues such as liver, brain, endothelial cells, heart, kidney, spleen, lung, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone, and skin (79, 80). At the time it is not known whether polyphenols accumulate to these target organs also in humans. Some compounds with long half-life, such as flavonols, may accumulate in blood when consumed repeatedly (81).

Excretion of polyphenols

Polyphenols and their metabolites are excreted either in urine or bile (40). Small conjugates are suggested to be excreted in the urine while large conjugates are excreted in the bile. Compounds excreted via biliary route into the duodenum can be deglycosylated by bacterial enzymes to be reabsorbed. The excreted amount in the urine varies between <1 to 30% of the ingested amount depending on the compound (64). It is characteristic for flavonols that their elimination is relatively slow when compared with the other polyphenols, half-lives ranging from 11 h to as long as 28 h (64). On the other hand, the excretion of catechins is rapid. The excretion is very low for

anthocyanidins (<1%), but higher for ferulic acid (27%) and cocoa epicatechins (30%) (40). Low excretion suggests that anthocyanidins are either poorly absorbed or extensively metabolized. The excretion is probably underestimated for some compounds, such as anthocyanidins, and all metabolites may not have been identified. Chlorogenic acid is extensively metabolized and only traces are found in urine, while 1/3 of caffeic acid ingested is found in urine. (68).

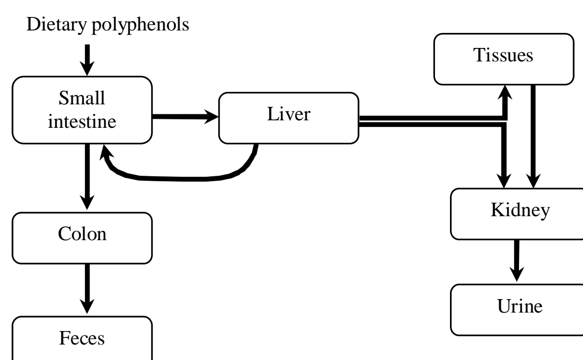

Figure 2. The main metabolic routes of polyphenols.

2.3 Polyphenols and cardiovascular health

The main interest assessing the health effects of polyphenols has focused on CVD. Epidemiological studies have explored the relation between polyphenol intake and the risk of CVD (**Table 3**), while the *in vitro*, animal and clinical studies have mainly assessed the protective mechanism(s).

2.3.1 Epidemiological evidence

Earliest findings about the role of flavonoids in CVD published in 1993 stem from the Zutphen Elderly Study, a small cohort of Dutch men (10). In this study, Hertog et al. explored a relation between the intake of 5 flavonoids from flavonol and flavone subclasses and the risk of CVD. They found that after 5 years of follow-up, the intake of flavonoids was associated with 68% [95% confidence interval (CI) 29-85%] reduced risk of CHD mortality after adjustment for CHD risk factors. Weaker inverse association was also found for the incidence of myocardial infarction (MI). Later, Keli and colleagues found that flavonoid intake was associated with 73% (95% CI 30-89%) reduced risk of fatal or non-fatal stroke (82). Later, Hertog and co-workers repeated their analyses for CHD mortality and found similar associations (83). Arts et al. assessed the role of 6 catechins from the flavan-3-ol subgroup in the same study and found catechin intake to be inversely

associated with 51% (95% CI 22-73%) reduced risk of CHD mortality (55). For MI or stroke no associations were found.

The role of flavonoids has been assessed also in the Finnish Mobile Clinic Health Examination Survey, a cohort with long follow-up time; up to 28 years. In the earliest findings, the intake of 5 flavonoids (flavonols and flavones) was found to be associated with 33% (95% CI 0-66%) reduced risk of CHD mortality in men, but not in women (84). Later, Knekt and co-workers studied the role of quercetin on the incidence of cerebrovascular disease, but did not find any association (85). In a more recent study, flavonoid intake was associated with a reduced risk of cerebrovascular disease, especially thrombotic stroke (54). More specifically, inverse associations were found for one flavonol (kaempferol) and two flavanones (hesperetin and naringenin). Quercetin was also found to be inversely associated with the ischemic heart disease (IHD) mortality.

The role of flavonoids in CVD has also been studied in the US in the large Health Professionals Follow-up Study. In their study Rimm and colleagues did not find flavonoid intake (flavonols and flavones) to be associated with the risk of CHD mortality (58).

Hertog et al. studied the relation between flavonols and IHD mortality in the Caerphilly Study, a small cohort consisting of men who were residents of Caerphilly, South Wales (57). In contrast to the previous findings, the flavonol intake was found to be associated with an increased risk of the all-cause mortality (rate ratio (RR), 1.4 [95% CI 1.0-2.0]). The authors speculated that this unexpected finding could be related to the fact that in the United Kingdom (UK) tea, which was the main source of flavonoids, is consumed with milk which could inhibit the absorption of flavonoids. Later studies, however, have not supported this hypothesis (86). Residual confounding by lifestyle factors may also have affected the results.

Yochum et al. found in the large Iowa Women Health Study that flavonoid intake was inversely associated with the risk of CHD death in postmenopausal women, while no association was found for stroke (59). In the further analysis of the same cohort, Arts et al. included also catechins from flavan-3-ol subclass (6 compounds), but did not find them to be associated with the CHD mortality (60). Recently, Mink et al. evaluated the role of 7 subclasses and found flavanones, anthocyanidins, and flavonoid rich foods to be inversely associated with total, CHD, and CVD mortality (87).

The role of flavonoids has also been studied in another Finnish cohort, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study. In their study, Hirvonen and co-workers did not find the intake to be associated with the risk of stroke in male smokers (88). In the same study, flavonoid intake was found to be associated with a significantly reduced risk of MI, but not with

the CHD mortality (53). More recently, a trend for an inverse association between flavonol and flavone intake and a risk of intermittent claudication was found (89).

In the Rotterdam Study, flavonol intake was found to be inversely associated with the fatal, but not with nonfatal, MI (56). Sesso et al. studied the relation between flavonoids and CVD in the large Women's Health Study, but did not find the flavonoid intake to be associated with the risk of CVD (61).

Hertog and colleagues evaluated the role of flavonoids in CHD in the cross-cultural Seven Country Study (90). Study consisted of total of 12 763 men aged 40 to 59 from 16 different cohorts. The study population included men living in Finland, Italy, Greece, former Yugoslavia, Japan, US and Netherlands. In a cross-sectional analysis, the flavonoid intake explained 9% of the total variance in CHD mortality. In another cross-sectional analysis consumption of flavonoid rich foods was inversely related with the risk of CVD (91).

Huxley and Neil gathered the data published before September 2001 and evaluated the association between flavonol intake and the risk of CHD (13). This meta-analysis consisted of 7 prospective cohorts and included a total of 105 000 people and 2087 fatal CHD events. After the adjustment for CHD risk factors, and dietary factors related to CHD, individuals in the top thirds of intake had a 20% reduced risk (95% CI 7-31%) as compared to those in the lower thirds.

Several case-control studies have also evaluated the role of polyphenols in CVD. Lagiou et al. found the intake of flavan-3-ols was associated with the decreased risk of CHD (62) and later peripheral arterial occlusive disease (92) in a Greek study. Marniemi and co-workers found the intake of two flavonoids, luteolin and kaempferol, to be inversely associated with the risk of acute myocardial infarction (AMI) in a Finnish population (93). Recently, the high intake of anthocyanidins was found to be related with the decreased risk of AMI (94).

The relation between the intake of phenolic acids and a CVD risk has not been explored, but numerous studies have assessed the role of coffee which is a rich source of phenolic acids. Similarly, tea is a rich source of flavan-3-ols and the role of tea in CVD has been extensively studied in epidemiological studies. Even though these beverages contain a mixture of compounds (polyphenols, caffeine etc.), these studies can provide information about the role of phenolic compounds in CVD. Red wine was not included in this review as the distinction of the effects of polyphenols from ethanol, which is known to affect the risk of CVD, is difficult.

The effects of coffee consumption on the risk of CVD have been studied for over 40 years. In the first study published in 1963, the high consumption of coffee (≥5 cups/d) was found to increase the risk of MI (95). Even though the association was later found to be explained solely by cigarette smoking, the study launched a series of further studies. A meta-analysis of 11 prospective studies

published between 1966 and 1991 revealed no association between coffee consumption and the risk of CHD (96). Studies conducted after the meta-analysis have resulted in inconsistent findings. Some have reported the high intake to be associated with an increased risk (97-100), and some have not found an association (101-103), while in some studies moderate consumption of coffee have been found to be beneficial (104-106). In some studies, the association has been J-shaped or U-shaped (107-109).

Similar to coffee, the CVD effects of tea have been evaluated for over 40 years. A meta-analysis, which included 10 cohort and 7 case-control studies published between 1966 and 2000, found an 11% decrease in the risk of MI for moderate tea consumers (3 cups or 700 ml/day) (110). The studies conducted after the meta-analysis have resulted in similar findings. Most studies have suggested moderate to high consumption of tea to be negatively related with CVD (56, 111, 112), although this has not been found in all studies (113).

In summary, despite the inconsistency, the results from prospective cohort studies suggest that flavonoids may modestly decrease the risk of CHD. Out of the 13 published studies, 8 longitudinal studies have found flavonoids to be significantly associated with the decreased risk of CHD. The number of studies on stroke is limited and out of 8 studies, only 2 have found a significant inverse association. The high consumption of tea may also moderately decrease the risk of CVD, while the coffee consumption does not seem to have a strong effect on the risk.

Table 3. Prospective studies of flavonoid intake and the risk of CVD[1]

Study and reference	Country	Population	Flavonoid subclasses	Contrast (high vs. low intake) (mg/d)	Follow-up time (y)	Outcome	No. of cases	Adjusted RR (95% CI) (high vs. low)
Zutphen Elderly Study Hertog et al. (1993) (10)	NL	805 M, 65-84 y	Flavonols, flavones	41.6 vs. 12.0	5	CHD mortality Fatal+non-fatal MI	43 38	0.32 (0.15-0.71) 0.52 (0.22-1.23)
Zutphen Elderly Study Keli et al. (1996) (82)	NL	552 M, 50-69 y	Flavonols, flavones	33.3 vs. 14.2	15	Fatal and non-fatal stroke	42	0.27 (0.11-0.70)
FMCHE Survey Knekt et al. (1996) (84)	FI	2745 M, 2380 F, 30-69 y	Flavonols, flavones,	> 4.8 vs. < 2.1 > 5.5 vs. < 2.4	26	CHD mortality	324 149	0.67 (0.44-1.00) 0.73 (0.41-1.32)
Health Professional Follow-up Study Rimm et al. (1996) (58)	US	34 789 M, 38 036 M, 40-75 y	Flavonols, flavones	40.0 vs. 7.1 40.0 vs. 7.1	6	Nonfatal MI CHD death	486 140	1.08 (0.81-1.43) 0.77 (0.45-1.35)
Caerphilly Study Hertog et al. (1997a) (57)	UK	1900 M, 45-59	Flavonols	42.8 vs. 13.5	14	CHD	131	1.60 (0.90-2.90)
Zutphen Elderly Study Hertog et al. (1997b) (83)	NL	804 M, 65-84 y	Flavonols, flavones	41.6 vs. 12.0	10	CHD	90	0.47 (0.27-0.82)
Iowa Women's Health Study Yochum et al. (1999) (59)	US	34 492 F, 55-69 y	Flavonols, flavones	28.6 vs. 4.0	10	CHD mortality Stroke mortality	438 131	0.62 (0.44-0.87) 1.18 (0.70-2.00)
FMCHE Survey Knekt et al (2000) (85)	FI	9208 M+F	Quercetin (flavonol)	>4.6 vs. <2.0 >5.2 vs. <2.3	28	Thrombotic stroke	M445 F378	0.99 (0.71-1.38) 0.85 (0.60-1.21)
ATBC Study Hirvonen et al. (2000) (88)	FI	26 497 M, 50-69 y	Flavonols, flavones	16.4 vs. 4.2	6	Stroke	736	0.98 (0.80-1.21)
Zutphen Elderly Study Arts et al. (2001a) (55)	NL	806 M, 65-84 y	Flavan-3-ols	124.0 vs. 25.3	10	CHD mortality MI Stroke	90 90 88	0.49 (0.27-0.88) 0.70 (0.39-1.26) 0.92 (0.51-1.68)
Iowa Women's Health Study Arts et al. (2001b) (60)	US	32 857 F	Flavan-3-ols	74.8 vs. 3.6	13	CHD	767	0.85 (0.67-1.07)
ATBC Study Hirvonen et al. (2001) (53)	FI	25 372 M, 50-69 y	Flavonols, flavones	17.8 vs. 3.9	6	Nonfatal MI CHD	1122 815	0.77 (0.64-0.93) 0.89 (0.7-1.11)
Rotterdam Study Geleijnse et al. (2002) (56)	NL	4807 M+F, ≥55 y	Flavonols	40.0 vs. 16.8	5.6	Nonfatal MI Fatal MI	116 30	0.93 (0.57-1.52) 0.93 (0.13-0.98)
FMCHE Survey Knekt et al (2002) (54)	FI	9131 M+F	Flavonols, flavones, flavanones	M > 26.9 vs. < 4.3 F > 39.5 vs. < 8.5	28	CHD Stroke	681 806	0.93 (0.74-1.17) 0.79 (0.64-0.98)
Women's Health Study Sesso et al. (2003) (61)	US	38 445 F	Flavonols, flavones	47.4 vs. 8.9	7	Total CVD Stroke, MI, CVD	729 519	0.88 (0.68-1.14) 0.80 (0.59-1.09)

[1]ATBC=Alpha-Tocopherol, Beta-Carotene Cancer Prevention, CI=confidence interval, CHD=coronary heart disease, CVD=cardiovascular disease, F=females, FI=Finland, FMCHE=Finnish Mobile Clinic Health Examination, M=males, MI=myocardial infarction, NL=Netherlands, RR=rate ratio, UK=United Kingdom, US=United States.

2.3.2 Mechanisms of actions

The mechanism(s) through which flavonoids may decrease the risk of CVD is not currently known. Flavonoids have e.g. antioxidant, vasodilatory, antithrombotic and anti-inflammatory properties, which may account for their protective effects.

Antioxidant effects

The most popular hypothesis for the protective mechanism of polyphenols against CVD is their ability to act as antioxidants. Polyphenols have been suggested to decrease the oxidative stress in human body and especially inhibit the oxidation of LDL (114). Flavonoids may inhibit the oxidative stress by: 1) scavenging free radicals by acting as reducing agent, hydrogen atom donating molecules or singlet oxygen quenchers; 2) chelating metal ions; 3) sparing other antioxidants (e.g. β-carotene, vitamin C and E); and 4) preserving HDL-associated serum paraoxonase activity (114). Antioxidant properties of polyphenols are related to their chemical structure and dependent on the number and arrangement of their phenolic hydroxyl groups (39, 115, 116).

In vitro studies about antioxidant effects of polyphenols

Large number of papers on the antioxidant properties of various polyphenols *in vitro* has been published and most of the compounds have been found to be powerful antioxidants. The evidence is especially extensive for monomeric (catechins) and polymeric (proanthocyanidins) flavan-3-ols. Antioxidant properties have been shown for pure compounds (117, 118) as well as food items rich in flavan-3-ols, mainly for black tea (118-121), green tea (118, 119, 122, 123), red wine (8, 119, 124), cocoa (125-128), and chocolate (127, 128). Also flavonols, especially quercetin, have been found to be effective antioxidants *in vitro* (129, 130). Antioxidant properties of other flavonoids or phenolic acids *in vitro* have been studied much less, but most of the compounds are considered to possess antioxidant activity (116). Various fruits and vegetables, which are rich sources of polyphenols, also have antioxidant activity (131).

In vivo relevancy of these *in vitro* experiments has been, however, questioned because the bioavailability and metabolic aspects have often not been taken into consideration (15, 132). Firstly, the concentrations of polyphenols which prevent oxidation *in vitro* have often been tens of times higher as compared to those achieved in the human body. The concentrations required to induce beneficial effect in *in vitro* range from <0.1 to >100 µmol/l, while the concentrations measured in the human body have usually been ~1 µmol/l after supplementation. Secondly, in

humans polyphenols are extensively metabolized after the absorption and thus the chemical forms that occur in foods, and often used *in vitro* studies, very rarely exist as such in the human body.

Animal studies about antioxidant effects of polyphenols

Animal studies have provided relatively strong and consistent evidence of the antioxidant and cardioprotective effects of polyphenols. In large number of studies, supplementation with polyphenols has inhibited oxidative stress (133-143) and attenuated the progression of atherosclerosis (133-136, 138, 140-142, 144-146). The beneficial effects on oxidative stress or atherosclerosis have mainly been found for various sources of monomeric and polymeric flavan-3-ols; green and black teas (134, 136-139, 144), red wine polyphenols (140, 142, 145-147) and cocoa (141). However, not all studies have found polyphenols to be beneficial in the inhibition of oxidative stress (145, 146, 148) or atherosclerosis (149-152). Studies of the role of polyphenols other than flavan-3-ols in animal models are limited. Some studies have also suggested flavonols, especially quercetin, to be able to inhibit lipid peroxidation and attenuate atherogenesis in animals (143, 147). No changes in atherogenesis have been detected after anthocyanidins or black currant juice supplementation (150).

Human supplementation studies about antioxidant effects of polyphenols

The antioxidant effects of polyphenols have been studied in humans since the mid 1990s. As in the case of *in vitro* and animal studies, these supplementation studies have focused on green and black tea, red wine and cocoa (15). These studies can be categorized into two types; studies which have assessed the effects of one bolus for 1 to 6 hours, and studies which have assessed relatively long-term effects, mainly from 2 to 4 weeks. The supplemented amount of polyphenols has varied from a few tens of milligrams to grams. In this review, the focus is in assessing the effects polyphenols and thus only those red wine studies which were controlled for alcohol were included.

In majority of the studies, the polyphenol supplementation has increased antioxidant capacity **(Tables 4-6)**. Enhanced capacity has been found for green tea and black tea (117, 122, 153-158), red wine polyphenols (154, 159-161), grape juice (162) and cocoa polyphenols (163-166). In addition, limited evidence suggests that other sources of polyphenols such as onions rich in quercetin (167) and coffee rich in phenolic acids may enhance the capacity (168). The increase in antioxidant capacity has lasted for a few hours, usually being highest at 1 to 2 hours after consumption of polyphenols.

In contrast, studies which have evaluated the effects of supplementation on lipid peroxidation have resulted in more inconsistent findings. Black tea or green tea have inhibited lipid peroxidation

in some (118, 156, 157, 169-171), but not in all studies (119-121, 123, 155). Similar discrepancy concerns studies on red wine polyphenols and cocoa. Some studies have found red wine polyphenols to decrease the oxidative stress (160, 162, 172-177), while there are also several studies which have not detected an effect (159, 178-180). For cocoa polyphenols, majority of studies have suggested inhibition of lipid peroxidation (126, 163, 165, 166, 181-185), while some studies have not found an effect (186-188). Limited amount of evidence suggests that other rich sources of polyphenols, such as berries, may also decrease lipid peroxidation (131). Limited amount of evidence support the putative decreasing role of quercetin in lipid peroxidation (175), even though not all studies have detected an effect (167).

More specifically, the number of studies which have used F_2-isoprostanes, which are considered to be the most reliable marker of oxidative stress *in vivo* (31-33), are much more limited. Majority of these studies have not detected beneficial effect on F_2-isoprostanes for tea (189-192), cocoa polyphenols (163, 183, 186, 187), grape juice (162), fruit juice mixture (193) or fruit rich diet (194). In some studies, decreased production of F_2-isoprostanes has been detected after consumption of red wine polyphenols (173, 195), cocoa (181) or diet rich in fruit and vegetables (196).

In summary, even though the results are inconsistent, the evidence favours the hypothesis that polyphenols enhance antioxidant capacity, while the evidence for inhibitory effects on lipid peroxidation *in vivo* is inconsistent. Some criticism against the beneficial effects of polyphenols has also been raised. Plasma total antioxidant capacity in human body is estimated to be $>10^3$ μmol/l and in order to detect a significant change in the capacity, the minimum increase in the polyphenol concentration of plasma should be at magnitude of 20-50 μmol/l (14, 197). The supplementation has, however, increased the concentrations of polyphenols in plasma mainly 1 μmol/l, and this has been suggested to be insufficient to exert biological effects. Alternatively, some have suggested that postprandial changes are results of increased concentration of metabolic antioxidant, uric acid (197).

Table 4. The main supplementation studies assessing the effects of cocoa polyphenols on antioxidant capacity and lipid peroxidation[1]

Reference	Design	Duration	N, gender	Contrast and daily dose	Measurement and outcome
Kondo et al. 1996 (126)	1 dose	4 h	12 M	Cocoa (35 g)	Ox-LDL ↓
Wang et al. 2000 (163)	1 dose	6 h	20 M+F	HP chocolate (0 g, 27 g, 53 g or 80 g)	Antioxidant capacity (↑) MDA (↓) F_2-isoprostanes −
Rein et al. 2000 (165)	1 dose	6 h	13 M+F	Chocolate (80 g) vs. vanilla milk chips	Antioxidant capacity (↑) MDA (↓)
Serafini et al. 2003 (164)	Crossover	4 h	12 M+F	DC (100 g) vs. DC (100 g) + milk (200 g) vs. milk chocolate (200 g)	Antioxidant capacity (↑) for DC
Wiswedel et al. 2004 (181)	Crossover	4 h	20 M	HP cocoa (100 g) vs. LP cocoa (100 g)	Antioxidant capacity − F_2-isoprostanes ↓
Vlachopoulos et al. 2005 (188)	Crossover	3 h	17 M+F	Chocolate (100 g) vs. water	Antioxidant capacity − MDA −
Osakabe et al. 2000 (182)	Parallel	2 wk	15 M	Cocoa (36 g) vs. sugar	Ox-LDL ↓
Wan et al. 2001 (166)	Crossover	2 wk	23 M+F	Cocoa (22 g) + DC (16 g)	Antioxidant capacity (↑) Ox-LDL ↓
Mathur et al. 2002 (183)	Uncontrolled	6 wk	25 M+F	DC (37 g) + cocoa (31 g)	Ox-LDL ↓ F_2-isoprostanes −
Murphy et al. 2003 (187)	Randomized	28 d	32 M+F	Cocoa polyphenol tablets (234 mg) vs. placebo	Antioxidant capacity − F_2-isoprostanes −
Engler et al. 2004 (186)	Randomized	2 wk	21 M+F	HP chocolate (46 g) vs. LP chocolate (46 g)	Antioxidant capacity − Ox-LDL − F_2-isoprostanes −
Fraga et al. 2005 (184)	Randomized	2 wk	28 M	HP chocolate (105 g) vs. LP chocolate (105 g)	TRAP − MDA ↓
Baba et al. 2007 (185)	Randomized	12 wk	25 M+F	Cocoa (26 g) + sugar (12 g) vs. sugar (12 g)	Ox-LDL ↓

[1] ↓=decrease, −=no effect, ↑=increase, ()=nonsignificant change. Beneficial change in antioxidant capacity ↑, beneficial change in lipid peroxidation ↓
DC=dark chocolate, F=females, HP=high polyphenol, LDL=low-density lipoprotein, LP=low polyphenol, M=men, MDA=malondialdehyde, Ox-LDL=oxidation susceptibility of LDL, TRAP=total radical trapping antioxidant parameter.

Table 5. The main supplementation studies assessing the effects of tea polyphenols on antioxidant capacity and lipid peroxidation[1]

Reference	Design	Duration	N, gender	Contrast and daily dose	Measurement and outcome
Serafini et al. 1996 (122)	1 dose, parallel	80 min	10 M+F	BT (300 ml) vs. GT (300 ml)	Antioxidant capacity ↑ (BT and GT)
Ishikawa et al. 1997 (118)	Randomized	4 wk	22 M	BT (750 ml) vs. water	Ox-LDL ↓
van het Hof et al. 1997 (119)	Parallel	4 wk	48 M+F	BT (900 ml vs. GT (900 ml) vs. water	Ox-LDL −
McAnlis et al. 1998 (121)	1 dose	3 h	5 M+F	BT (600 ml) vs. coffee	Antioxidant capacity −
	Randomized	1 wk	10 M+F	BT (1500 ml) vs. coffee	Ox-LDL −
Princen et al. 1998 (123)	Randomized	4 wk	64 M+F	BT (3 g of solids) vs. GT (3 g of solids) vs. GTE (3.6 g)	Ox-LDL −
Benzie et al. 1999 (158)	1 dose	2 h	12 M+F	GT (400 ml) vs. water	Antioxidant capacity ↑
Cherubini et al. 1999 (120)	1 dose	3 h	8 M+F	Tea (500 ml)	Ox-LDL −
Freese et al. 1999 (170)	Randomized	4 wk	20 F	GT extract (3 g) vs. placebo	MDA ↓
Hodgson et al. 2000 (171)	Crossover	1 h	20 M	BT (7.6 g of tea leaves) vs. GT vs. caffeinated water vs. water	Antioxidant capacity − Serum-Ox ↓ for BT (↓) for GT
Leenen et al. 2000 (153)	1 dose, crossover	2 h	21 M+F	BT (2 g of solids) vs. GT (2 g of solids) vs. water	Antioxidant capacity ↑ (BT and GT)
Serafini et al. 2000 (154)	OSSS	2 h	5 M+F	BT (300 ml) vs. GT (300 ml) vs. water	Antioxidant capacity ↑ (BT and GT)
Hodgson et al. 2002 (189)	Crossover	1 wk	13 M+F	BT (1000 ml) vs. GT (1000 ml) vs. water	F_2-isoprostanes −
	Crossover	4 wk	22 M+F	BT (1250 ml) vs. water	F_2-isoprostanes −
Young et al. 2002 (155)	Crossover	3 wk	16 M	GT + meat patties vs. meat patties	Antioxidant capacity ↑ Serum-Ox −
Henning et al. 2004 (117)	Crossover	8 h	30 M+F	BT (4 bags) vs. GT (3 bags) vs. GTE (3 capsules)	Antioxidant capacity ↑ (GTE)
Donovan et al. 2005 (192)	Uncontrolled	14 d	9 M+F	GTE (4 capsules)	F_2-isoprostanes −
Erba et al. 2005 (156)	Uncontrolled	42 d	24 F	GT (640 mg)	Antioxidant capacity ↑ MDA ↓
Reddy et al. 2005 (157)	OSSS	3 d	9 M	BT (7 g of tea leaves) vs. BT (7 g of tea leaves) + milk	Antioxidant capacity ↑ MDA ↓
Widlansky et al. 2005 (190)	Crossover	2 h	66 M+F	BT (450 ml) vs. water	Antioxidant capacity −
	Crossover	4 wk	66 M+F	BT (900 ml) vs. water	F_2-isoprostanes −
Coimbra et al. 2006 (169)	OSSS	4 wk	34 M+F	GT (1000 ml) vs. water	Antioxidant capacity − MDA ↓

[1] ↓=decrease, −=no effect, ↑=increase, ()=nonsignificant change. Beneficial change in antioxidant capacity ↑, beneficial change in lipid peroxidation ↓
BT=black tea, F=females, GT=green tea, GTE=green tea extract, LDL=low-density lipoprotein, MDA= malondialdehyde, M=men, OSSS=open series supplementation study, Ox-LDL=oxidation susceptibility of LDL, Serum-Ox=oxidation susceptibility of serum.

Table 6. The main supplementation studies assessing the effects of red wine polyphenols on antioxidant capacity and lipid peroxidation[1]

Reference	Design	Duration	N, gender	Contrast and daily dose	Outcome
Kondo et al. 1994 (177)	OSSS	2 wk	10 M	RW (0.8 g/kg) vs. ethanol (0.8 g/kg)	Ox-LDL ↓
Fuhrman et al. 1995 (174)	Parallel	2 wk	17 M	RW (400 ml) vs. WW (400 ml)	MDA ↓ Ox-LDL ↓
de Rijke et al. 1996 (178)	Randomized	4 wk	24 M+F	RW (550 ml) vs. WW (550 ml)	MDA − Ox-LDL −
Carbonneau et al. 1997 (159)	OSSS	2 wk	20 M	RWP (6 capsules)	Antioxidant capacity ↑ Ox-LDL −
Miyagi et al. 1997 (176)	Parallel	2 h	20 M+F	RW (300 ml) vs. WW (450 ml) vs. grape juice (300 ml)	MDA ↓ RW
Nigdikar et al. 1998 (172)	Parallel	2 wk	39 M	RW (375 ml) vs. WW (375 ml) vs. WW + RWP (375 ml) vs. RWP (1 g) vs. ethanol (400 ml)	MDA ↓ in groups 1, 3, 4 Ox-LDL ↓ in groups 1, 3, 4
	Parallel	2 wk	20 M	RWP (1 g) vs. RWP (2 g)	MDA ↓ in both groups Ox-LDL ↓ in both groups
Caccetta et al. 2000 (179)	Randomized	4 h	12 M	RW vs. PSRW vs. DRW vs. water (5 ml red wine equivalents/kg)	Ox-LDL − Serum Ox −
Chopra et al. 2000 (175)	Randomized	2 wk	21 M	RWP (1 g) vs. quercetin	Ox-LDL ↓ in both groups
Serafini et al. 2000 (154)	OSSS	2 h	5 M+F	DRW (300 ml) vs. DWW (300 ml)	Antioxidant capacity ↑
Caccetta et al. 2001 (173)	Randomized	2 wk	18 M	RW (375 ml) vs. WW (375 ml) vs. DRW (500 ml)	F_2-isoprostanes ↓ for DRW
Natella et al. 2001 (160)	OSSS	3 h	6 M	RW (400 ml) vs. ethanol (400 ml)	Antioxidant capacity ↑ Ox-LDL ↓
Cartron et al. 2003 (180)	Crossover	3 wk	18 M	RW (300 ml) vs. WW (300 ml) vs. champagne (300 ml)	Antioxidant capacity ↓ MDA −
Arendt et al. 2005 (198)	1 dose	6 h	27 M+F	RW (200 ml) vs. DRW (175 ml)	Antioxidant capacity −
	Randomized	6 wk	78 M+F	Water (200 ml) vs. RW (200 ml) vs. DRW (175 ml)	Antioxidant capacity −
Modun et al. 2006 (161)	1 dose, crossover	3 h	9 M	RW vs. PSRW vs. ethanol vs. water (3 ml/kg)	Antioxidant capacity ↑ (RW and PSRW)
Pignatelli et al. 2006 (195)	Randomized	15 d	20 M+F	RW (300 ml) vs. WW (300 ml)	F_2-isoprostanes ↓

[1]↓=decrease, −=no effect, ↑=increase. Beneficial change in antioxidant capacity ↑, beneficial change in lipid peroxidation ↓
D=dealcoholized, F=females, M=men, LDL=low-density lipoprotein, MDA=malondialdehyde, Ox-LDL=oxidation susceptibility of LDL, OSSS=open series supplementation study, P=polyphenols, RW=red wine, S=stripped, WW=white wine.

Vasodilatory effects on endothelial function

The vascular endothelium plays a key role in the regulation of vascular homeostasis and accumulating evidence shows that endothelial dysfunction contributes to the pathogenesis of CVD (199, 200). Oxidative stress and impaired endothelial function are closely related, and therefore antioxidants, such as flavonoids, could also have an effect on endothelial function (201). Several studies have found a beneficial effect of different flavonoids and flavonoid-rich foods on endothelial function in humans. Beneficial effects have been observed for flavan-3-ols rich foods; purple or red grape juice (202, 203), red wine (204) or red wine polyphenols (205), tea (190, 206-208), and for cocoa polyphenols (186, 188, 209-212). Even though not all studies have found beneficial effects for tea or red wine polyphenols (204, 213), the findings have been relatively consistent. Flavonoids are proposed to improve endothelial function e.g. by increasing the production of NO (201). NO has vasodilatory, anti-inflammatory, antithrombotic, and growth-suppressing properties.

Antithrombotic and anti-inflammatory properties

Polyphenolic compounds have been suggested to have anti-inflammatory and antithrombotic properties. Platelet aggregation is a key factor in CVD and antiplatelet therapy reduces the risk. Limited evidence suggests that consumption of grape juice, red wine polyphenols (214) cocoa polyphenols (187, 214-217), black or green tea may inhibit platelet aggregation. Not all studies have supported the hypothesis (166).

Atherosclerosis is a chronic inflammatory disease (16). Some (217), but not all studies (183, 190) have provided evidence of the anti-inflammatory properties. In addition, limited amount of evidence suggests that polyphenols may decrease blood pressure (210, 218, 219) and increase insulin sensitivity (210, 219). Originally, it was suggested that polyphenols could have LDL lowering properties, but studies have not supported this hypothesis. On the other hand, limited evidence suggests that cocoa polyphenols may increase HDL cholesterol (166, 185), but majority of studies have not found such an effect.

2.3.3 Safety of polyphenol consumption

Some concerns about the safety of polyphenol consumption have been raised. The consumption of polyphenols is known to inhibit the absorption of non-heme iron and thus high intake of polyphenols could be one contributing factor to developing anemia in those with marginal iron stores (220).

Consumption of coffee increases circulating levels of tHcy in humans, which has been suggested to be an independent risk factor for CVD (221). Caffeine and chlorogenic acid have been found to be responsible for the tHcy increase (222, 223), but the public health relevancy of these findings remains a subject of argument.

Polyphenols may also affect the bioavailability and pharmacokinetics of certain drugs. Consumption of naringenin rich grapefruit has been found to inhibit cytocrome P450 3A4 mediated metabolism of several drugs (224). Grapefruit has also been found to increase the bioavailability of some benzodiazepines (225), lipid lowering drugs (226), and possibly thyroid medicines (227). Orange juice has also been found to decrease the mean peak concentration of beta-blockers in plasma (228).

In addition, polyphenols among the other antioxidants are considered to be potential pro-oxidants when administrated in high amounts (34, 229). Some studies have reported pro-oxidant properties of polyphenols *in vitro* (230). In humans, no increasing effects on lipid peroxidation have been verified probably due to considerably lower amounts ingested and the extensive metabolism of polyphenols.

Despite the fact that evidence does not support the adverse effects of polyphenols on humans, some caution, especially with the dosing, is needed. The safety of supraphysiological doses, such attainable through dietary supplements, can not be guaranteed. Swiss physician and chemist Paracelsus in 17th century stated “All substances are poisons…it is the dose that distinguishes a poison from a remedy.”

2.4 Summary of the literature review

In summary, polyphenols are a large group of compounds ubiquitous in plants. In humans, the daily intake of polyphenolic compounds is estimated to vary from ten milligrams to one gram. Polyphenols are absorbed into human body, but for most of the compounds the bioavailability is relatively poor. Epidemiological evidence suggests that high intake of flavonoids may modestly decrease the risk of CHD. However, the evidence concerns mainly flavonols, and the role of other polyphenols in CVD has been studied much less. In addition, because of limited amount of studies, the evaluation of the role of polyphenols in strokes is premature. The most popular explanation for their mechanism of action is related to the antioxidant properties of polyphenols. Polyphenols are strong antioxidants *in vitro*, but the effects observed in humans are inconsistent.

3. AIMS OF THE STUDY

In general, the aim of this study was to assess the role of dietary polyphenols in CVD. First, the aim was to explore relations between polyphenol intake and atherosclerosis, and the risk of CVD in eastern Finnish middle-aged men. Second, the aim was to explore the suggested protective mechanism of action by studying the effects of polyphenol supplementation on oxidative stress in humans.

The specific aims of the present work were:

I **To explore the relation between dietary flavonoid intake and common carotid artery intima-media thickness (CCA-IMT) in middle-aged men.**

II **To study the association between dietary flavonoid intake and the risk of cardiovascular diseases in middle-aged men.**

III **To study the effects of supplemented polyphenols in chocolate, coffee and phloem, on oxidative stress in humans.**

4. MATERIALS AND METHODS

4.1 The Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study (Works I and II)

4.1.1 Study population (Works I and II)

The KIHD Study is an on-going population-based study of risk factors for CVD, atherosclerosis, and related outcomes in men from Eastern Finland (231). The baseline examinations were carried out between March 1984 and December 1989. The study sample consisted of 3235 men aged 42, 48, 54, or 60 years at baseline examination. Of these, 2682 (82.9 %) participated. The study protocol was approved by the Research Ethics Committee of the University of Kuopio. All subjects gave their written informed consent for participation. The cross-sectional work I included 1380 men for who complete data, including the CCA-IMT measurements, were available. For the work II men who had history of CHD or stroke were excluded and a complete data were available for 1950 men.

4.1.2 Measurement of CCA-IMT (Work I)

In work I, CCA-IMT was assessed by high-resolution B-mode ultrasonography of the right and left CCAs at the distal end, proximal to the carotid bulb. The ultrasound equipment (Biosound Phase 2; Biosound Inc, Indianapolis, US) was equipped with a high-resolution probe. Images were focused on the posterior wall of the right and left CCAs and were recorded on videotape for image analysis. The ultrasonographic examinations were carried out by well-trained ultrasound technicians and were performed after the subjects had rested in a supine position for 15 min.

CCA-IMT measurements were made through computerized analysis of the videotaped ultrasound images with PROSOUND software (University of Southern California, Los Angeles, US). This software uses an edge-detection algorithm, specifically designed for use with ultrasound imaging, that allows automatic detection, tracking, and recording of the intima-lumen and media-adventitia interfaces, estimated at ≈100 points, in both the right and left CCAs in a 1.0–1.5 cm section (232). For the present study, 2 measures of CCA-IMT were used. Mean CCA-IMT was computed as the mean of ≈100 IMT measurements in the right CCA and another 100 measurements in the left CCA. A separate study concerning the intra- and interobserver variability of CCA-IMT measurements was carried out in 10 randomly chosen middle-aged men who had participated in the KIHD study. The between-observer coefficient of variation (CV) was 10.5% for the first assessments by 4 observers for both the right and left CCAs. The correlation coefficients

ranged from 0.90 to 0.99. The intraobserver variability was described by the absolute value of difference between the first and the third measurement by each observer. The mean absolute difference was 0.087 mm, which is 8.1% of the mean of all measurements (233).

4.1.3 Ascertainment of follow-up events (Work II)

For work II, the collection of data and the diagnostic classification of strokes between 1984 and 1992 were carried out as a part of the multinational World Health Organization (WHO) Monitoring of Trends and Determinants in Cardiovascular Diseases (MONICA) project, in which detailed information of strokes were collected prospectively (234). In the Finnish part of the WHO MONICA project (FINMONICA), regional coronary and stroke register teams collected data on strokes from hospitals and wards of health centers and classified the events, as explained in detail previously (235). Data on strokes from the beginning of 1993 were obtained by computer linkage to the national hospital discharge and death registers. Strokes were collected and classified by a neurologist using the same procedures as in the FINMONICA study (235). Each suspected stroke (International Classification of Diseases, ICD-9 codes 430-438 and ICD-10 codes I60-I68 and G45-G46) was classified into 1) a definite stroke, 2) no stroke or 3) an unclassifiable event. Each definite stroke was classified into 1) an ischemic stroke (ICD-9 codes 433-434, ICD-10 code I63) or 2) a hemorrhagic stroke (ICD-9 codes 430-43, ICD-10 codes I60-I61) (236).

CVD deaths were ascertained by computer linkage to the national death registry using the Finnish social security number. All CVD deaths that occurred from the study entry to 31 December 2004 were included. There were no losses to follow-up. CVD deaths were coded according to the 9th ICD (code numbers 390 to 459) and the 10th ICD (code numbers I00 to I99).

Definite ischemic strokes and CVD deaths were used as outcome events. If a subject had multiple strokes during the follow-up, the first was considered as the end point.

4.1.4 Other measurements (Works I and II)

For work I and II, the study subjects were instructed to abstain from ingesting alcohol for three days, from smoking and eating for 12 hours. Fasting blood samples were drawn between eight and ten o'clock in the morning after the subject had rested in the supine position for 30 minutes. Blood was drawn with Terumo Venoject (Leuven, Belgium) vacuum tubes without using a tourniquet.

The main serum lipoprotein fractions, LDL and HDL cholesterol (Kone Instruments, Espoo, Finland), and triglycerides (Boehringer Mannheim, Mannheim, Germany) were determined from fresh serum samples using combined ultracentrifugation and precipitation. Maximal oxygen uptake

was measured as previously described (237). Diabetes was assessed by previous diagnosis of diabetes or fasting blood glucose concentration ≥6.7 mmol/l. Resting systolic blood pressure was measured after supine rest of five minutes, three measurements in supine, one on standing and two in sitting position with five minutes' intervals with a random-zero mercury sphygmomanometer (Hawksley, UK). The mean of all six measurements was used as the systolic blood pressure. Body mass index (BMI) was computed as the ratio of weight to the square of height (kg/m^2). The number of cigarettes, cigars, and pipefuls of tobacco currently smoked daily, duration of regular smoking in years, alcohol consumption, history of myocardial infarction, angina pectoris, and medication were recorded with a self-administered questionnaire, which was checked by an interviewer. A subject was defined as smoker if he had ever smoked on a regular basis and had smoked cigarettes, cigars, or pipe within the past 30 days. Repeated interviews to obtain medical history of CHD were conducted by a physician. The family history of CHD was defined as positive if either father, mother, sister, or brother of the subject had a history of CHD.

4.1.5 Assessment of nutrient intakes (Works I and II)

The consumption of foods at the KIDH study baseline was assessed at the time of blood sampling with an instructed 4-day food recording by household measures. The instructions were given and the completed food records were checked by a nutritionist. The intakes of nutrients were estimated using the Nutrica® computer software version 2.5 software. The intakes of nutrients used as covariates in the Cox models were energy adjusted by the residual method (238). Energy adjustment is based on the notion that a larger, more physically active person requires a higher caloric intake, which is associated with a higher absolute intake of all nutrients. Therefore energy adjustment takes into account differences in energy requirements among individuals. The residuals were standardized by the mean nutrient intake of a subject consuming 10 MJ/d, the approximate average total energy intake in this study population.

The measurement of total, subclass and individual flavonoid intake in work I and II was based on United States Department of Agriculture (USDA) flavonoid database (239). Database includes total of 26 flavonoids from 5 subclasses; flavonols (quercetin, kaempferol, myricetin, isorhamnetin), flavones (luteolin, apigenin), flavanones (hesperetin, naringenin, eriodictyol), flavan-3-ols ((+)-catechin, (+)-gallocatechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin-3-gallate, (-)-epigallocatechin-3-gallate, theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate, theaflavin-3,3'-digallate, thearubigins) and anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin). The USDA database is incomplete for antocyanidin-rich berries

which are commonly consumed in Finland. Therefore, additional anthocyanidin data for those Finnish berries were derived from the work conducted by Kähkönen et al. (240).

4.1.6 Statistical analyses (Works I and II)

In work I, correlations between the intakes of flavonoids and other risk factors with CCA-IMT were estimated with Pearson correlation coefficients (*r*). The heterogeneity of the means of baseline variables between the quarters of total flavonoid intake was tested by using analysis of variance (ANOVA). Frequency distribution of the categorical variables between quarters of total flavonoid intake was compared by the χ^2 test. Baseline risk factors used as covariates in the ANOVA included age and technical covariates (examination years and baseline zooming depth given separately for right and left side), history of atherosclerosis, smoking, BMI, diabetes, systolic blood pressure, serum HDL and LDL cholesterol, maximal oxygen uptake, and intakes of alcohol, saturated fat (percent of energy, E%), and energy adjusted intakes of folate, vitamin C and E. The linear trend across flavonoid quarters was tested using ANOVA.

In work II, the subjects were classified into quarters according to their energy adjusted flavonoid intake. The relationship of flavonoid intake with the risk of CVD was analyzed using Cox proportional hazards models. Model 1 was adjusted for age and examination years, and model 2 in addition for BMI, systolic blood pressure, serum total, HDL and LDL cholesterol, serum triglycerides, maximal oxygen uptake, smoking, family history of ischemic heart disease, diabetes, alcohol intake, saturated fat intake (percent of energy, E%) and energy adjusted intake of fiber, vitamin C and E. RRs adjusted for other risk factors, were estimated as antilogarithms of coefficients for independent variables. The CIs were estimated based on the assumption of asymptotic normality of estimates. The means were compared using ANOVA and categorical variables using chi-square tests.

In works I and II the distributions were expressed as means and standard deviations (SD). All statistical tests were two-tailed. Data were analyzed using SPSS for Windows version 11.5 statistical software (SPSS Inc., Chicago, Illinois, US).

4.2 Clinical supplementation studies (Works III-V)

4.2.1 Study populations

Study subjects in work III consisted of 45 non-smoking men and women (26±9 years), in work IV of 45 non-smoking men (26±6 years) and in work V of 75 non-smoking men (51±11 years). For

works III-V potential participants were screened in an interview for the following inclusion criteria: 1) BMI<32 kg/m^2; 2) no regular use of any drugs or supplements with antioxidative (ß–carotene, vitamins C or E), or lipid lowering properties; 3) no chronic diseases such as diabetes, CHD or other major illness; and 4) willingness to consume study supplements; chocolate (work III), coffee (work IV) or phloem enriched rye bread (V). For work V, an additional criterion for the participation was elevated serum total cholesterol concentration (6-9 mmol/l). For all supplementation trials, the participants were recruited from the Kuopio area. Works III and IV were advertised in a local university student newspaper and via e-mail at the University of Kuopio and study V in a local newspaper. Informed consents were obtained in writing from all participants after they had read a description of the experimental procedures.

4.2.2 Study designs (Works III-V)

The study protocol of works III-V consisted of a run-in period and the supplementation period. The lengths of the period and the content of the nutritional restrictions differed between the works. The study protocols were approved by the Research Ethics Committee, Hospital District of Northern Savo.

Run-in period

For the run-in period of work III and V, subjects were advised to discontinue the use of tea, red wine, cocoa and chocolate one week prior to the supplementation period. In work IV the duration of the run-in period was two weeks and during this period the use of coffee, tea, red wine, cocoa and chocolate was prohibited. In addition, the intake of fruit- and berry-derived juices was restricted to a maximum of 300 ml (2 glasses) per day. Subjects were given caffeine tablets to be used if necessary for the withdrawal symptoms. The maximum daily amount of caffeine was the amount comparable to that obtained from the daily study bolus in the long-term study (0, 300 or 600 mg). In all of these works, subjects were also advised to avoid the use of alcohol and analgesics for three days and vigorous physical activity for one day before visits. In all works these study specific restriction were instructed to be maintained throughout the supplementation period.

Supplementation period

The chocolate study (work III) was a 3-week clinical supplementation study with 3 parallel groups. Participants consumed daily 75 g of either white chocolate (white chocolate group, WC group), dark chocolate (dark chocolate group, DC group), or dark chocolate enriched with cocoa polyphenols (high polyphenol chocolate, HPC group). In order to enhance study compliance, the

subjects were allowed to choose the study group. The daily amount of flavan-3-ols in the study chocolates were <1 mg, 273 mg and 418 mg in the WC, DC, and HPC groups, respectively. The study chocolates were delivered by Oy Karl Fazer Ab, Vantaa, Finland and Meiji Seika Kaisha Ltd., Chiyoda Sakado-shi Saitama, Japan.

The coffee study (work IV) was 3-week clinical supplementation study in which subjects consumed daily either 0, 450 ml (3 cups) or 900 ml (6 cups) of filtered coffee. The study was not randomized for the same reasons as in the work III. Ingested amounts of phenolic compounds through coffee were 0, 364 and 728 mg/d in the 0, 3 and 6 cup groups, respectively. The short-term study in work IV was conducted directly after obtaining the blood samples for the supplementation period of the long-term study. The subjects remained in the same group as in the long-term study, but consumed a single dose of 1/3 of the total daily dose consumed in the long-term study (0, 1 or 2 cups, 0, 150 or 300 ml, respectively). Blood sample was taken 1.5 hours after coffee ingestion. The coffee used in this study was commercial finely ground coffee packed in 500 g packages and delivered by Oy Paulig Ab, Helsinki, Finland.

The phloem study (work V) was a 4-week randomized double-blind supplementation study. Subjects were randomly allocated to consume daily 70 grams of normal dried rye bread (placebo group, n=30), rye bread in which 7% of the rye flour was substituted with phloem powder (low polyphenol, LP group, n=30) or bread in which 14% of the rye flour was substituted with phloem powder (high polyphenol, HP group, n=15). Study was conducted in two parts; for the first part 15+15 men were recruited to the placebo and for the LP groups. To test the effects of higher amount of polyphenols in the second part, 15+15+15 men were recruited to the placebo, LP and HP groups. The placebo group received 0.6 mg, LP group 30.8 mg and HP group 62.0 mg of flavan-3-ols daily from the study bread.

In works III-V blood, samples were drawn with Venoject vacuum tubes (Terumo) after an overnight fast (10 hours). In work IV, subjects collected also a 24-h urine sample prior to the study visits. All measurements were done at the baseline and after the supplementation period. In all works (III-V) a four-day food recording was required before and during the last week of the supplementation period to check the compliance of the given instructions. The intakes of nutrients were analyzed by using the Nutrica® software (version 2.5).

The protocol of the study visits in supplementation studies was identical. During the first visit before the trial the subjects were given information about the study, and if the consent form was signed, more specific instructions were given. During the following study visit, a fasting blood sample was drawn, the 4-day food record was checked with nutritionist, instructions concerning the

study were repeated and the study preparation was given. At the end of the study, a fasting blood sample was drawn and the food record was checked with the nutritionist.

4.2.3 Measurements of oxidative stress

Plasma free F_2-isoprostanes (Works III and IV)

A deuterated prostaglandin $F_{2\alpha}$ internal standard was added to the plasma, and free F_2-isoprostanes were extracted using C_{18} and silica mini-columns. The compounds were converted to pentafluorobenzyl ester trimethylsilyl ether derivates and analyzed by a GC-MS assay (Agilent Technologies, Espoo, Finland) (30).

Plasma hydroxy fatty acids (Works III and IV)

In works III and VI, plasma C_{18} hydroxy fatty acids were measured using a CG-MS (Agilent Technologies, Espoo, Finland) (35). Shortly, plasma fatty acids and fatty acid hydroperoxides were stabilized by hydrogenation using platinum as a catalyst, saponified, esterified by diazomethane, and finally, in order to separate hydroxy fatty acids from fatty acids, extracted by solid phase mini-columns. Prior to the analysis, hydroxy groups were methylated with tetramethylammonium hydroxide. Concentrations of different (methoxy) OHFA methyl esters were determined by electron impact mass spectrometer. C17 and C19 OHFAs were used as internal standards.

Serum LDL conjugated dienes (Works III and IV)

The oxidation of LDL *in vivo* was assessed in work III and IV by following the formation of conjugated dienes as described previously (241). In brief, serum LDL was isolated by precipitation with buffered heparin. The precipitate was re-suspended in phosphate-buffered saline (PBS). Cholesterol concentration was determined and the rest of the suspension was used for conjugated diene measurement. Lipids were extracted from the LDL by mixture of chloroform and methanol (3:1), dried under nitrogen, re-dissolved in cyclohexane, and the amount of conjugated dienes was measured spectrophotometrically at 234 nm and 300 nm. Absorbance at 300 nm was subtracted from that at 234 nm. The conjugated diene concentration was calculated per cholesterol concentration in LDL.

Oxidation resistance of serum lipids and very-low-density lipoprotein (VLDL)+LDL (Works III-V)

The resistance of serum lipids to oxidation was measured as described previously (242). Briefly, serum was diluted to a concentration of 0.67% in 0.02 mol/l PBS, pH 7.4. Oxidation was initiated by the addition of 100 µl of 1 mmol/l $CuCl_2$ to 2 ml of diluted, pre-warmed (30°C) serum. The formation of conjugated dienes was observed by monitoring the change in the absorbance of 234 nm at 30°C on a Beckman DU-640i spectrophotometer (Fullerton, California, US) equipped with a six-position automatic sample changer. The change in absorbance was recorded every 5 min for 4 hours. The time required from the start to reach the maximal rate of the reaction (lag time) was determined.

In the work V, the oxidation resistance of VLDL+LDL fraction was also studied. Briefly, VLDL and LDL were isolated in a combined fraction from fresh ethylenediamine tetraacetic acid (EDTA) plasma by ultracentrifugation. EDTA and gradient salts were removed by gel permeation columns, and VLDL+LDL was exposed to copper-induced oxidation and the lag time was determined as previously described (241).

Plasma TRAP (Work III)

In work III, plasma TRAP was determined with a modification of the method originally published by Metsä-Ketelä (28, 241). Briefly, radical generator was added into luminol containing buffer and the production of peroxyl radicals in the thermal decomposition of 2,2'-azobis (2-amidinopropane) hydrochlorine was followed with a luminometer (Model 1251, Bio Orbit Oy, Turku, Finland) at 32°C. Plasma sample was added after 15 min. The TRAP value was calculated from the duration of the disappearance of chemiluminecence.

Activity of antioxidant enzymes (Work IV)

Plasma GPX in work III was determined by a commercial kit (Ransel RS 505, Randox Laboratories, San Diego, California, US) by using Konelab 20 Analyzer (Thermo Clinical Labsystems, Vantaa, Finland). Serum paraoxonase (PON) activity was measured from serum based on its capacity to hydrolyze paraoxon. The formation of *p*-nitrophenol was monitored at 405 nm in Tris-HCl buffer, pH 8.0, in the presence of Ca^{2+} (243).

Effects of the phloem breads and catechin on serum lag time in vitro (Work V)

To study the effects of phloem polyphenols on the oxidation resistance of total serum lipids *in vitro*, the breads were ground and 100 mg were extracted 3 times with 3 ml of 80% MeOH. Supernatants were taken into 10 ml volumetric flask after the centrifugation and flask was filled with 80% MeOH. Into five different vials were taken 0, 10, 50, 100 and 200 µl of phloem bread extract which was then evaporated under N_2 flow. The dry residue was dissolved in control serum, which was diluted to concentration of 0.67% with PBS buffer, at 30°C. The oxidation was initiated by the addition of 1 mmol/l $CuCl_2$. The formation of conjugated dienes was followed as described above. The effect of catechin was studied with a pure compound in a separate assay. The *in vitro* concentrations of phloem derived catechins in serum ranged from 0.01 to 0.24 µg/ml for placebo bread, from 0.88 to 17.6 µg/ml for LP bread, and from 1.77 to 35.4 µg/ml for HP bread. The *in vitro* concentration of catechin standard in serum ranged from 2.0 to 39.9 µg/ml.

1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging capacity of study breads (Work V)

The radical scavenging capacity of study breads against DPPH• radical was analysed as follows; extracts of the study breads were prepared by an ultra-sonication-assisted extraction with 50% MeOH. The concentration of the extracts obtained was 250 mg raw material/ml solvent. These extracts were diluted in MeOH into a range of concentrations to enable determination of IC_{50}value. 600 µl of DPPH• solution (60 µM in MeOH) was added to 600 µl of each diluted sample and the resulting solution was allowed to react for 30 min in the dark at ambient temperature. The absorbance caused by the DPPH• radical at 517 nm was determined by Unicam UV 500 Spectrophotometer (Unicam, UK) as described earlier (244, 245). Radical scavenging capacity is expressed as $1/IC_{50}$ and values are the means of 3 replicates.

4.2.4 Other laboratory measurements

Serum lipoproteins (Works III-V)

In works III-V, the serum cholesterol (Konelab, Espoo, Finland) and triglycerides (Roche Diagnostics, Mannheim, Germany) were determined using enzymatic colorimetric tests. The serum concentration of HDL cholesterol was measured on supernatant after magnesium chloride dextran sulphate precipitation. Serum LDL cholesterol was determined by a direct cholesterol measurement (Konelab, Espoo, Finland).

Serum and LDL fatty acids (Works III-V)

Serum and LDL fatty acids were analyzed in work III and IV after extraction using chloroform-methanol and methylation with sulphuric acid-methanol. The methylated fatty acids were analyzed by a gas chromatograph (Hewlett Packard 5890, Avondale, Pennsylvania, US) equipped with a flame ionization detector and an NB-351 capillary column (HNU-Nordion, Helsinki, Finland) (246). Serum LDL was isolated by precipitation with buffered heparin. The precipitate was re-suspended in PBS (247). The concentration of cholesterol was determined and the rest of the suspension was used for measuring LDL conjugated diene and LDL fatty acids. The fatty acids of serum and LDL are presented as percentages of the total amount of fatty acids analyzed.

Plasma tHcy (Work IV)

In work IV, plasma tHcy concentration was analyzed by high performance liquid chromatography (HPLC) as described previously (248). The CV between batches (n=30) for two pooled plasma samples were 5.7% (7.3 µmol/l) and 7.1% (10.5 µmol/l).

Plasma folate and vitamin B_{12} (Work IV)

In work IV, plasma folate and vitamin B_{12} were measured simultaneously by radioimmunoassay (Quantaphase II, Bio-Rad, Hercules, California, US).

Safety measurements (Works III-V)

In works III-V blood cell profile, including erythrocyte, leukocyte and thrombocyte counts and hemoglobin, was measured by a blood cell counter (Advia 60, Bayer, Tarrytown, New York, US). Serum aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) were measured with a clinical chemistry analyzer (Konelab, Espoo, Finland). The activity of serum gamma-glutamyltransferase (γ-GT) was measured with the International Federation of Clinical Chemistry method (249).

4.2.5 Measurements of polyphenol content of the study supplements and biological samples

Catechin and procyanidin content of the study supplements (Works III and V)

Flavan-3-ol content of the study preparations were analysed with a method modified from the method originally published by Arts and co-workers (250). Analyses were carried out using an HPLC with a coulometric electrode array detector (ESA Inc. Chelmsford, Massachusetts, US). Analysed catechins included 7 compounds; (+)-catechin, (-)-catechin-gallate, (-)-gallocatechin, (-)-

epicatechin, (-)-epicatechin-3-gallate, (-)-epigallocatechin, and (-)-epigallocatechin-3-gallate and 3 procyanidin dimers; epicatechin-(4β→8)-catechin (B1), epicatechin-(4β→8)-epicatechin (B2), and catechin-(4α→8)-epicatechin (B4). The samples were analyzed in triplicate.

Phenolic acid analyses of coffee and urine (Work IV)

Phenolic acid analyses were carried out with an HPLC using a coulometric electrode array detector. Compounds were separated with gradient elution using end-capped C 18 column Inertsil ODS-3 (150 x 3 mm) packed with 3 µm particles and C 18 guard column (10 x 3 mm, 5 µm particles). Mobile phase consisted of eluent A) 50 mM KH_2PO_4 / H_3PO_4 buffer pH 2.3 : MeOH 90:10 (v/v) and B) 50 mM KH_2PO_4 / H_3PO_4 buffer pH 2.3 : MeOH :ACN 40:40:20 (v/v/v). Chlorogenic, caffeic, ferulic, *p*-, *m*-, o-coumaric, sinapinic, protocatechuic and gallic acid were measured from coffee and from urine additionally two metabolites 3,4-dihydroxyphenyl- and *m*-hydroxyphenylacetic acids were measured. Coffee analysis was carried out after the combination of the enzyme and base hydrolysis. 24-h urine samples were hydrolyzed with β-glucuronidase and sulphatase obtained from *Helix pomatia*. Hydrolyzed samples were extracted with diethyl ether, evaporated under N_2 flow and dissolved in MeOH. Samples were diluted with eluent prior to HPLC run.

4.2.6 Statistical methods in supplementation studies (Works III-V)

In works III-V, the means across the study groups were compared by the ANOVA. Post-hoc Tukey's test was used whenever a statistically significant heterogeneity between the groups was shown by the ANOVA. *P*-value ≤ 0.05 was considered significant. Simple correlation and stepwise linear regression analysis were used to estimate the contribution of changes in the formation of conjugated dienes in work III. Differences between baseline and end-point values within the pooled groups were tested by paired t test. All tests of significance were two-sided. The results are expressed as means±SD. Data were analyzed using SPSS 10.0, 11.0 or 11.5 for Windows (SPSS Inc., Chicago, Illinois, US).

5. RESULTS

5.1 The intake of flavonoids and carotid atherosclerosis (Work I)

In this cross-sectional study, the mean CCA-IMT was 0.78±0.17 mm. Mean intake of flavonoids was 128.5±206.7 mg/d and each subclass contributed to the total intake as follows: flavan-3-ols 84% (107.7 mg/d); flavonols 7% (9.1 mg/d); anthocyanidins 6% (7.5 mg/d); flavanones 3% (3.9 mg/d) flavones less than 1% (0.3 mg/d). The intake of flavonoids was associated with healthy lifestyle; men with the high intake were less likely to be smoker, had lower intakes of alcohol, total fat, and SAFA, but had higher intakes of folate, fiber, vitamin C and E (**Table 7**).

Table 7. Characteristics of the 1380 study subjects and according to the quarters of energy-adjusted flavonoid intake[1]

		Quarters of flavonoid intake (mg/d)				
	All	1 (lowest)	2	3	4 (highest)	*P*[2]
CCA-IMT (mm)	0.78±0.17	0.79±0.19	0.81±0.18	0.77±0.16	0.76±0.15	<0.001
Flavonoid intake (mg/d)	128.5±207.7	3.6±1.8	18.2±7.2	85.9±37.5	406.4±253.7	<0.001
Flavonoid intake (mg/d)[3]	128.5±206.7	0.0±16.4	26.4±8.8	89.8±35.6	404.9±250.3	<0.001
Age (y)	52.4±6.4	51.6±6.3	53.6±6.3	52.4±6.3	52.0±6.6	<0.001
Body mass index (kg/m^2)	26.6±3.5	26.5±3.7	26.7±3.6	26.8±3.4	26.6±3.5	0.739
SBP (mmHg)	132±17	132±16	133±17	131±17	133±16	0.426
S-LDL chol (mmol/l)	3.94±0.96	3.98±0.90	4.00±1.06	3.80±1.03	3.97±0.84	0.020
S-HDL chol (mmol/l)	1.29±0.30	1.31±0.28	1.29±0.32	1.30±0.30	1.27±0.29	0.291
S-triglycerides (mmol/l)	1.42±0.86	1.33±0.73	1.46±0.94	1.47±0.91	1.44±0.84	0.097
VO_{2max} (ml/kg/min)	30.6±7.4	31.5±7.3	29.6±7.2	30.9±7.4	30.5±7.4	0.006
Smokers (%)	39.6	52.5	44.1	35.4	26.7	<0.001
Total fat intake (g/d)[3]	99.2±17.2	101.7±21.4	101.4±15.8	96.5±15.2	97.4±14.9	<0.001
SAFA intake (g/d)[3]	45.2±11.5	46.6±14.3	46.9±10.5	43.8±10.0	43.7±10.3	<0.001
Alcohol intake (g/d)[3]	11.8±20.2	14.0±26.9	11.3±18.6	11.8±16.7	10.1±16.6	0.087
Folate intake (µg/d)[3]	256±61	234±59	247±54	269±61	276±62	<0.001
Fiber intake (g/d)[3]	25.4±8.9	24.0±9.6)	25.0±6.8	26.5±7.7	26.2±7.4	<0.001
Vitamin C intake (mg/d)[3]	71.2±51.0	51.8±34.3	65.8±45.1	86.4±55.3	80.9±58.5	<0.001
Vitamin E intake (mg/d)[3]	9.2±2.5	8.8±2.8	8.9±2.4	9.4±2.3	9.5±2.6	<0.001

[1]Mean±SD. CCA-IMT=common carotid artery intima-media thickness, Chol=cholesterol, HDL=high-density lipoprotein, LDL=low-density lipoprotein, S=serum, SAFA=saturated fatty acids, SBP=systolic blood pressure, SD=standard deviation, VO_{2max}=maximal oxygen uptake.
[2]*P* value from the ANOVA (continuous variables) or *P* value from chi-square test (discrete variables).
[3]Intakes of nutrients are energy adjusted (238).

In the covariance analysis after multivariate adjustment, total flavonoid intake was inversely associated with the mean CCA-IMT (*P*=0.018) (**Table 8**). Out of 5 subclasses, flavan-3-ols were

inversely associated with CCA-IMT (*P*=0.046) after identical statistical adjustment. A nonsignificant trend was found for flavonols and the mean CCA-IMT (*P*=0.055, after statistical adjustment). The intake of other subclasses; flavones (*P*=0.505), flavanones (*P*=0.875), and anthocyanidins (*P*=0.577) were not associated with the mean CCA-IMT.

TABLE 8. CCA-IMT of the 1380 study subjects according to the energy-adjusted quarters of flavonoid intake[1]

	Quarters of flavonoid intake (mg/d)				
	1 (lowest)	2	3	4 (highest)	*P*[2]
Total flavonoid intake (mg/d)[‡]	<12.5	12.5-43.7	>43.7-166.3	>166.3	
CCA-IMT (mm)	0.79 ± 0.19	0.81 ± 0.18	0.77 ± 0.16	0.76 ± 0.15	0.018
Flavonol intake (mg/d)	<3.7	3.7-6.6	>6.6-11.9	>11.9	
CCA-IMT (mm)	0.81 ± 0.20	0.79 ± 0.17	0.77 ± 0.16	0.76 ± 0.15	0.055
Flavone intake (mg/d)	0.0	>0.0-0.1	>0.1-0.3	>0.3	
CCA-IMT (mm)	0.76 ± 0.17	0.80 ± 0.19	0.79 ± 0.17	0.77 ± 0.16	0.505
Flavanone intake (mg/d)	0.0	>0.0-0.1	>0.1-0.5	>0.5	
CCA-IMT (mm)	0.76 ± 0.17	0.79 ± 0.17	0.80 ± 0.19	0.77 ± 0.16	0.875
Flavan-3-ol intake (mg/d)	<0.2	0.2-20.3	>20.3-140.6	>140.6	
CCA-IMT (mm)	0.78 ± 0.17	0.81 ± 0.19	0.78 ± 0.17	0.76 ± 0.15	0.025
Anthocyanidin intake (mg/d)	0.0	>0.0-1.3	>1.3-5.6	>5.6	
CCA-IMT (mm)	0.78 ± 0.17	0.78 ± 0.17	0.79 ± 0.17	0.78 ± 0.18	0.577

[1]Mean±SD. CCA-IMT=common carotid artery intima-media thickness, SD=standard deviation.
[2]Adjusted P value from ANOVA. Adjusted for age, examination years, baseline zooming depth given separately for right and left side, history of atherosclerosis, smoking, BMI, diabetes, systolic blood pressure, serum HDL and LDL cholesterol, maximal oxygen uptake, and intakes of alcohol, saturated fat (percent of energy, E %), and energy adjusted intakes of folate, vitamin C and E.

5.2 The intake of flavonoids and the risk of ischemic stroke and CVD death (Work II)

The mean flavonoid intake was 139.3mg/d and out of total intake, flava-3-ol subclass contributed 85.9% (119.7 mg/d), flavonols 7.2% (10.0 mg/d), anthocyanidins 4.5% (6.2 mg/d), flavanones 2.2% (3.1 mg/d), and flavones 0.2% (0.3 mg/d). The mean age of the study population was 52.4±5.3 years and during the average follow-up time of 15.2 years, men with no previous CHD or stroke, experienced 102 ischemic strokes and 153 CVD deaths. As in work I, high intake of flavonoids tended to be associated with healthier lifestyle. When comparing in quarters of flavonoid intake, the men with the highest quarter of intake were more likely to be nonsmoker, tended to have lowest alcohol and SAFA intakes and higher intakes of fiber, vitamin C and E, when compared with the lower quarters.

In a Cox proportional hazards model adjusted for age and examination years, the RRs for the intakes of flavonol and flavan-3-ols subclasses were 0.52 (95% CI 0.30-0.90) in the highest quarter

of flavonol intake versus the lowest, and for flavan-3-ols 0.52 (95% CI 0.28-0.99), respectively (**Table 9**). After multivariate adjustment, the RRs for the ischemic stroke in the highest quarter of flavonol and flavan-3-ol intakes were 0.54 (95% CI 0.30-0.97) and 0.54 (95% CI 0.28-1.04), respectively. The intakes of other subclasses; anthocyanidins, flavanones or flavones were not associated with the risk of ischemic stroke. None of the subclasses were associated with the CVD mortality.

Table 9. Relative risks (95% CIs) of CVD according to the quarters of energy-adjusted flavonoid intake[1]

	Quarters of flavonoid intake (mg/d)			
	1 (lowest)	2	3	4 (highest)
Ischemic stroke				
Flavonols	1	0.68 (0.40-1.14)	0.53 (0.30-0.93)	0.54 (0.30-0.97)
Flavones	1	1.12 (0.60-2.10)	1.91 (1.08-3.37)	1.16 (0.62-2.18)
Flavanones	1	0.82 (0.46-1.45)	0.96 (0.55-1.68)	0.90 (0.49-1.66)
Flavan-3-ols	1	1.21 (0.71-2.05)	0.98 (0.56-1.72)	0.54 (0.28-1.04)
Anthocyanidins	1	0.88 (0.48-1.63)	1.58 (0.92-2.71)	0.91 (0.49-1.68)
Total sum of flavonoids	1	1.62 (0.96-2.74)	1.04 (0.57-1.88)	0.67 (0.35-1.29)
CVD mortality				
Flavonols	1	1.29 (0.82-2.04)	1.27 (0.79-2.03)	1.13 (0.67-1.88)
Flavones	1	0.81 (0.50-1.32)	0.88 (0.55-1.39)	0.74 (0.46-1.19)
Flavanones	1	0.66 (0.40-1.07)	1.15 (0.74-1.77)	0.63 (0.39-1.04)
Flavan-3-ols	1	1.33 (0.85-2.09)	1.05 (0.65-1.68)	0.99 (0.60-1.63)
Anthocyanidins	1	0.67 (0.40-1.15)	1.33 (0.85-2.07)	1.16 (0.73-1.85)
Total sum of flavonoids	1	1.91 (1.23-2.97)	1.04 (0.63-1.72)	1.15 (0.69-1.91)

[1]Adjusted for age, examination years, BMI, systolic blood pressure, serum total, HDL and LDL cholesterol, serum triglycerides, maximal oxygen uptake, smoking, ischemic heart disease in family, diabetes, alcohol intake, saturated fat intake and energy adjusted intake of fiber, vitamin C and E. CI=confidence interval, CVD=cardiovascular disease.

5.3 The effects of chocolate on HDL cholesterol and lipid peroxidation (Work III)

All 45 recruited volunteers completed the study and no adverse effects were reported by the study subjects or found in the laboratory analyses (ASAT, ALAT or γ-GT) (**Table 10**). Mean weight decreased during the study in the WC group (-1.1±2.7 kg) and increased in the DC group (0.4±0.7 kg) and in the HPC group (0.8±0.9 kg) (P<0.05, between study groups). During the study, the total energy intake and the proportion of fat and saturated fat in the diet increased, whereas the proportion of protein and carbohydrates decreased. No differences between the study groups were found in the intake of nutrients. The compliance with the nutritional instructions was good and none of the subjects reported consumption of the restricted foods.

Table 10. Baseline values and changes after 3 week consumption of study chocolates[1]

	WC (n=15)		DC (n=15)		HPC (n=15)		
	Baseline	Change	Baseline	Change	Baseline	Change	P^2
BMI (kg/m^2)	22.3±2.3	-0.4±1.0	21.5±2.9	0.1±0.2	24.1±3.5	0.3±0.3	0.012
S-ASAT (U/l)	23±7	-2±7	22±8	2±12	20±6	4±9	0.230
S-ALAT (U/l)	24±13	-1±15	17±8	-2±7	16±9	4±18	0.478
S-γ-GT (U/l)	15±7	0±5	14±6	-0±3	18±11	1±7	0.741
S-creatinine (µmol/l)	85±11	0±9	79±7	1±6	84±11	2±7	0.842
S-total chol (mmol/l)	5.21±0.72	-0.02±0.51	4.74±0.90	0.08±0.49	4.99±1.01	0.12±0.47	0.710
S-LDL chol (mmol/l)	2.80±0.57	0.17±0.60	2.57±0.68	0.00±0.37	2.82±0.62	0.00±0.39	0.627
S-HDL chol (mmol/l)	1.49±0.32	-0.00±0.14	1.41±0.38	0.14±0.15	1.38±0.29	0.18±0.12	<0.001
S-triglyceride (mmol/l)	1.45±0.74	-0.15±0.59	1.12±0.55	-0.21±0.46	0.95±0.35	0.00±0.49	0.336
P-TRAP (µmol/l)	1057±206	22±134	973±176	85±250	1155±170	92±229	0.657
S-lipid oxidation resistance (lag time, min)	118±43	6±31 (14)	122±35	4±32 (14)	160±61	-3±28 (14)	0.711
S-LDL conjugated dienes (µmol/mmol chol)[3]	16.3±3.1	-4.0±6.9	16.7±2.6	-5.9±7.6	15.5±2.7	-4.6±5.7	0.496
P-F_2-isoprostanes (pg/ml)	43.4±13.8	-2.5±7.9	48.7±22.0	-5.3±15.9	45.4±11.2	-0.9±8.1	0.554
P-OHFA (µmol/l)	1.02±0.47	-0.02±0.41	1.04±0.48	-0.05±0.48	1.08±0.46	-0.04±0.32	0.987

[1]Mean±SD. ALAT=alanine aminotransferase, ASAT=aspartate aminotransferase, BMI=body mass index, Chol=cholesterol, DC=dark chocolate, γ-GT=gamma-glytamyltransferase, HDL=high-density lipoprotein, HPC=cocoa polyphenol enriched dark chocolate, LDL=low-density lipoprotein, OHFA=hydroxy fatty acids, P=plasma, S=serum, SD=standard deviation, TRAP=total radical trapping antioxidant parameter, WC=white chocolate.
[2]*P* for the differences between the changes in the groups (one-way ANOVA).
[3]Significant differences between baseline and end-point values within the pooled groups ($P<0.001$, paired *t* test).

Consumption of study chocolates increased the serum HDL cholesterol concentration in the DC and HPC groups (11.4% and 13.7%, respectively), while a slight decrease was seen in the WC group (-2.9%) ($P<0.001$, between study groups). No changes were seen in serum total or LDL cholesterol or triglyceride concentrations.

The consumption of chocolates decreased lipid peroxidation as measured by the formation of conjugated dienes *in vivo*. The production decreased in all study groups by a mean 11.9% ($P<0.001$) with no difference between the groups. No changes were found in the antioxidant capacity (plasma TRAP), or the other markers of lipid peroxidation; oxidation susceptibility of serum lipids, plasma hydroxy fatty acids and F_2-isoprostanes.

5.4 The effects of coffee on lipid peroxidation and plasma tHcy (Work IV)

Out of 45 men recruited, 43 completed the long-term study; of these, 35 participated in the short-term study. One subject dropped out during the run-in period due to abstinence symptoms from coffee drinking, and one was excluded due to dizziness during the process of drawing the blood samples. At study baseline, age, BMI and the activities of ALAT and γ-GT enzymes were higher in the 900 ml group when compared with other groups (**Table 11**). The difference in the activity of ALAT in the 900 ml group was due to high activities in two persons (149 and 140 U/l). During the supplementation period no adverse effects because of coffee consumption were reported or detected in the safety measurements (ALAT, ASAT or γ-GT). The intake of nutrients did not differ between the groups.

The consumption of coffee increased the concentration of polyphenols and their metabolites in urine. At baseline total excretion of phenolic acids was 123 (0 ml), 109 (450 ml) and 101 (900 ml) μmol/d and 126, 152 and 157 μmol/d after the 3-week ingestion of coffee, respectively. The increases in the concentrations of caffeic, ferulic, protocatechuic and 3,4 dihydroxyphenylacetic acids were different between the study groups ($P<0.05$). The change in the total excretion of phenolic acids in 450 ml and 900 ml groups represented 3.8% and 2.5% of the daily-ingested amounts.

The consumption of filtered coffee did not have short- or long-term effects on serum lipids, lipid peroxidation or activity of antioxidant enzymes. Plasma tHcy concentration increased by 5%, 16% and 26% in the 0, 450 and 900 ml groups, respectively ($P=0.102$). Because the mean age and BMI differed between groups at the study baseline, we adjusted the change in the plasma tHcy for age, BMI and the baseline concentration of tHcy. After adjustment the trend in the difference of plasma tHcy change between study groups attenuated ($P=0.494$). The consumption of coffee did not have an effect on plasma concentrations of folate or B-12 vitamin.

Table 11. Baseline values and changes after 3 wk supplementation period[1]

	Daily coffee intake						
	0 ml (n=15)		450 ml (n=14)		900 ml (n=14)		
	Baseline	Change	Baseline	Change	Baseline	Change	P^2
S-LDL chol (mmol/l)	2.2±0.8	-0.0±0.3	2.3±0.6	-0.0±0.4	2.6±0.5	-0.0±0.5	0.948
S-HDL chol (mmol/l)	1.0±0.1	-0.0±0.1	1.1±0.2	0.0±0.1	1.1±0.2	0.0±0.1	0.324
S-triglycerides (mmol/l)	1.1±0.7	-0.0±0.6	1.1±0.6	-0.1±0.3	1.3±0.6	0.1±0.7	0.496
S-lipid oxidation resistance (lag time, min)	210±23	-14±29	200±43	6±46	210±54	-15±52	0.602
S-LDL conjugated dienes (μmol/mmol chol)	14.8±3.6	1.3±3.8	16.5±4.5	-0.3±4.3	16.0±2.7	-0.8±3.8	0.597
P-F_2-isoprostanes (pg/ml)	32.0±7.3	-0.1±4.9	30.1±3.0	1.7±7.4	31.6±9.4	-0.8±7.5	0.853
P-OHFA (μmol/l)	0.70±0.18	-0.01±0.25	0.77±0.18	-0.04±0.21	0.75±0.17	0.09±0.18	0.374
P-GPX (U/l)[2]	830.4±134.2	23.3±83.0	920.4±124.6	20.4±88.1	862.5±115.6	61.4±109.1	0.664
S-PON (U/l)[2]	105.3±63.2	-1.0±7.2	114.5±82.3	-0.6±7.9	114.3±82.6	2.4±13.0	0.542
P-folate (nmol/l)	6.7±2.1	-0.4±1.3	7.8±2.2	0.1±3.9	6.5±1.6	-0.4±0.8	0.593
P-B_{12} (pmol/l)	368.9±242.9	28.6±210.8	436.9±154.5	29.1±116.2	369.3±88.1	-21.1±75.2	0.955
P-tHcy (μmol/l)[2]	11.4±9.4	0.5±1.2	8.6±2.2	1.4±1.9	11.7±3.5	3.0±5.0	0.494

[1]Mean±SD. Chol=cholesterol, DC=dark chocolate, GPX=glutathione peroxidase, HDL=high-density lipoprotein, HPC=cocoa polyphenol enriched dark chocolate, LDL=low-density lipoprotein, OHFA=hydroxy fatty acids, P=plasma, PON=paraoxonase, S=serum, tHcy=total homocysteine.
[2]*P* for differences in the changes between the groups. In the ANOVA age, body mass index at baseline and the baseline value of the parameter tested were used as a covariate.

5.5 The effects of polyphenol-rich phloem on lipid peroxidation in men (Work V)

All the 75 volunteers completed the study. Two participants were excluded, one in the placebo group due to an insufficient dietary compliance and one in the LP group due to a high concentration of serum triglycerides (8.8 mmol/l). At the study baseline, the activity of ASAT and ALAT enzymes were higher in the HP group when compared with the other groups ($P<0.05$) (**Table 12**). No other differences in the characteristics or the dietary intake of nutrients were found between the groups at the entry. According to food records and the questionnaire, the compliance of the volunteers to the given dietary and lifestyle instructions was good. No adverse effects were reported by the subjects during the study.

An increase in the oxidation resistance of total serum lipids measured as a lag time to maximal oxidation rate was observed in the HP group (11.4±13.8%, $P<0.01$, between study groups), while no change was seen in the LP group or in the placebo group. There were no significant differences in the changes of the oxidation resistance of VLDL+LDL between the study groups. The

consumption of placebo, LP or HP bread did not significantly alter serum lipids; total, LDL or HDL cholesterol or triglyceride concentrations.

Table 12. Baseline values and changes after 4 wk consumption of study breads[1]

	Placebo (n=29)		LP (n=29)		HP (n=15)		
	Baseline	Change	Baseline	Change	Baseline	Change	P^2
S-ASAT (U/l)	26±6	0±5	27±7	-0±6	31±9	-3±12	0.318
S-ALAT (U/l)	31±11	3±11	34±18	-1±15	44±20	-5±11	0.139
S-Creatinine (μmol/l)	92±15	-2±14	94±11	-0±7	93±10	7±8	0.037
S-LDL chol (mmol/l)	4.82±1.15	-0.20±0.59	4.96±0.92	-0.11±0.78	4.77±0.90	-0.12±0.58	0.886
S-HDL chol (mmol/l)	1.38±0.34	-0.02±0.15	1.26±0.24	0.00±0.15	1.19±0.23	0.02±0.24	0.776
S-triglycerides (mmol/l)	1.72±0.85	-0.18±0.75	1.74±0.92	0.24±0.68	2.23±1.37	-0.01±1.09	0.152
S-lipid oxidation resistance (lag time, min)	165±25	-3±17 (28)	180±22	-2±24	175±27	20±23 (12)	0.007
VLDL+LDL oxidation resistance (lag time, min)	64±5	3±7	66±6	2±9	66±6	-1±3 (14)	0.341

[1]Mean±SD. ALAT=alanine aminotransferase, ASAT=aspartate aminotransferase, HDL=high-density lipoprotein, HP=High polyphenol group, LDL=low-density lipoprotein, LP=low polyphenol group, S=serum, VLDL=very low-density lipoprotein.
[2]*P* for the differences in the changes between the study groups (one-way ANOVA).

The serum creatinine concentrations increased significantly in the HP group (7.8±10.1%, $P<0.05$), while no change was detected either in the LP group or in the placebo group. The change observed in the HP group was largely due to an increase in a single subject from 84 to 111 μmol/l. No other differences were detected in the laboratory measurements; blood cell count, hemoglobin, activity of ASAT, ALAT or γ-GT enzymes or concentrations of serum fatty acids between the study groups.

In the *in vitro* study, the phloem inhibited the oxidation of serum in a dose-dependent manner (**Figure 3**). After incubation of the placebo, LP and HP breads, the oxidation resistance of serum increased 18%, 90% and 137%, respectively. The pure (+)-catechin increased the oxidation resistance by 42%. $DPPH^{\bullet}$ also increased linearly with the amount of phloem in the study breads. The radical scavenging capacity of LP bread was 257% and HP bread 564% higher when compared with the placebo bread.

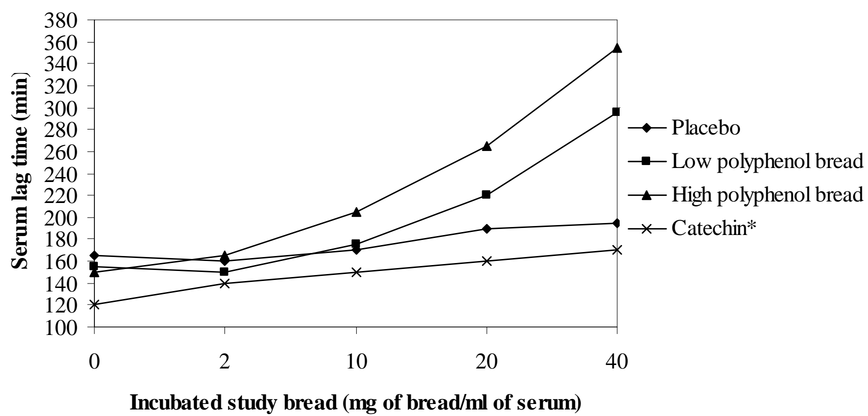

Figure 3.

The effects of study breads and catechin on the oxidation resistance of serum.
*Tests with catechin were conducted in a separate assay with different serum.

6. DISCUSSION

6.1 Methodological considerations

6.1.1 Study populations

The KIHD study population in the works I and II was selected using representative population sample of middle-aged eastern Finnish men. Participation rate was high with no losses to follow-up. Therefore, the results can be generalized for Finnish men, but not necessarily for women. However, studies have not suggested that the effects of flavonoids on the risk of CVD would differ between men and women. The reason for the selection of only men was the exceptionally high rates of CHD mortality among eastern Finnish men in the 1970s (251).

The study participants in the supplementation studies III and IV were healthy, young (~26 years), nonsmoking subjects, while work V consisted of older (~51 years), hypercholesterolemic, but otherwise healthy men. It is plausible that in order to benefit from antioxidant supplementation the objects should have an increased rate of oxidative stress as a result of e.g. smoking or subclinical disease (36, 252). Decreased lipid peroxidation was seen in older subjects in work V, while no effect was detected in younger subjects in works III and IV. It is possible that subjects in work V have suffered from increased oxidative stress, and did therefore benefit from antioxidant supplementation. However, this hypothesis is not supported by our findings showing that at baseline the oxidative stress (as measured as oxidation susceptibility of serum) was similar in the subjects participating in work V when compared with the studies subjects of works III and IV.

6.1.2 Study designs

In general, the main strength of the KIHD study is a large amount of high quality data which enables extensive adjustment for potential confounding factors. The main drawback of the work I was cross-sectional analysis and thus evaluation of temporal relations is not possible. In contrast, the work II was longitudinal study.

The supplementation work V was a randomized double-blind study, while works III and IV were not randomized. Parallel design was chosen to enhance study compliance. In work IV, those subjects who were not habitual coffee drinkers could have had difficulties to consume 900 ml of coffee per day. Similarly, in work III strong taste preferences on types of chocolate could have affected the study compliance.

Nonrandomized design resulted in selection bias in work IV. At the baseline, those in the group consuming the highest amount of coffee tended to be older and had higher BMI and activities of ALAT and γ-GT enzymes as compared to the groups which consumed either moderate amount or no coffee. To control for the potential confounding, these factors were included as covariates in the statistical models. The baseline values of the markers of oxidative stress, however, did not differ between the study groups. We believe that limitation in the study design did not have significant effect on the outcome in works III and IV.

In our supplementation works III-V we used flavan-3-ols (catechins and proanthocyanidins) and phenolic acids as sources of polyphenols. Flavan-3-ols were selected because they are considered to be especially powerful antioxidants. Phenolic acids, as well as caffeine and other methylxanthines have antioxidant activity, but so far the antioxidant effects of coffee *in vivo* have been studied only scarcely (168).

The supplemented amount of polyphenols in supplementation studies were 270 or 420 mg/d (work III), 365 or 730 mg/d (work IV), 30 or 60 mg/d (work V) and the duration of the supplementation periods were 3 (works III and IV) or 4 weeks (work V). We do think that the dose and the length of the supplementation should have been sufficient as studies with similar doses and durations have detected effects on oxidative stress. Supplemented amounts were also chosen to be comparable with the amount attainable from a habitual diet.

6.1.3 Measurements and collection of outcome events

Atherosclerosis in the work I was assessed by ultrasonography of the carotid arteries. IMT has been shown to be an independent predictor of cardiovascular disease (253, 254) and therefore non-invasive IMT measurements of the carotid arteries can be used as a valid indicator of atherosclerosis and the risk of CVD.

Data of ischemic strokes and CVD deaths in the work II is reliable and the risk of misclassification minimal. The collection of data and the diagnostic classification of strokes between 1984 and 1992 were carried out regional teams from hospitals and wards of health centers and classified the events as a part of the multinational WHO MONICA project (234, 235). Data on strokes from the beginning of 1993 were obtained by computer linkage to the national hospital discharge and death registers. CVD deaths were ascertained by computer linkage to the national death registry using the Finnish social security number.

The main strength of supplementation studies was that we used a wide set of markers of oxidative stress. We measured antioxidant capacity (work IV), various markers of lipid peroxidation; oxidation susceptibility of serum (III-V), oxidation susceptibility of LDL+VLDL

(V), conjugated dienes *in vivo* (III and IV), hydroxy fatty acids and F_2-isoprostanes *in vivo* (III and IV), and activity of antioxidant GPX and PON enzymes (IV). In addition, we assessed the antioxidant properties of administered breads *in vitro* in work V.

6.1.4 Dietary assessment and flavonoid database

In the KIHD study (works I and II), the dietary intake of flavonoids was assessed using 4-day food recording before the study visits. The intake of flavonoids may vary between different seasons, being highest in summer and autumn when vegetables are consumed in higher amounts. Seasonal variation may have caused some misclassification of subjects and may have caused underestimation in the relation between flavonoid intake and the CVD.

We used USDA database (52) which enabled to assess the role of a total of 26 compounds from 5 flavonoid subclasses. Earlier studies have included mainly 3 flavonols and 2 flavones, while the role of other subclasses has been studied much less (**Table 3**). It has been estimated that out of 11 to 26 identified subclasses of flavonoids at least 5 subclasses (flavonols, flavones, flavanones, flavan-3-ols, and anthocyanidins), a total of 20-30 compounds, may contribute significantly to the daily intake and thus also to CVD health (54).

Accurate data on the total intake is important in order to reveal the absolute strength of association. In addition, studying the role of each subclass and possibly individual compounds is important as chemical properties of the compounds differ and therefore possibly also the effects on health. However, this is complicated by the fact that many of the compounds occur in the same foods. For some subclasses, e.g. flavanones, separate analyses are possible as these compounds are almost solely derived from citrus fruits.

Even though we did study the effect of larger set of compounds, the database is still incomplete for anthocyanidins and procyanidins. This is mainly because the methods for the analysis of these compounds have been available only for a few years. Drawback of this study was that because of limitations in our computer software used to calculate the intakes of nutrients, we were not able to study food sources of flavonoids.

6.2 Dietary intake of flavonoids

The total intake of flavonoids was 129 mg/d (work I) and 139 mg/d (work II), and the flavan-3-ols were the main contributor accounting almost ~85% of the total intake. Because the USDA database included more compounds than used in most of the previous calculations the intake was higher than reported previously for Finnish (8-24 mg/d) or for the other populations (14-72 mg/d) (**Table 2**).

The KIHD study population consisted of middle-aged men who likely consumed low amounts of vegetables and thus the intake is probably lower than average population. In addition, the actual intake of some of the flavonoid subclasses is probably higher as databases are still incomplete. In addition to flavonoids, other polyphenolic compounds, such as phenolic acids, are ubiquitous in plant derived foods and have not been included in the calculations. Coffee is an especially rich source of phenolic acids (~100 mg/dl) and among coffee consumers the daily intake of phenolic acids alone may be around several hundreds of milligrams. Originally, Kühnau estimated in the mid 1970s that the daily intake of polyphenols would be around 1 gram, and this early estimation may eventually turn out to be surprisingly accurate (42).

6.3 Flavonoid intake, atherosclerosis and CVD

The main finding in the cross-sectional work I was that the high intake of flavonoids was associated with decreased carotid atherosclerosis as measured as CCA-IMT. In work II, the intake of flavonoids was associated with the decreased risk of ischemic stroke. However, for CVD death no such association was found.

Ischemic stroke and most of the other CVD stem from atherosclerosis, and thus the intake of flavonoids was expected to be associated also with CVD deaths. One possible explanation could be that atherosclerosis might lead to stroke earlier because of smaller diameter of the cerebral arterioles. Another explanation could be that despite similar etiology, some differences in the pathogenesis could explain the differences in the risks. Elevated blood pressure is the main risk factor for ischemic stroke, while its role in the other CVD is less strong. However, in our study the flavonoid intake was not associated with blood pressure and thus does not seem to explain the differences.

Our results are partly in line with the previous epidemiological findings. Out of 13 published cohort studies, 8 studies have found the high flavonoid intake to be significantly associated with the decreased risk of CHD (54, 82) (**Table 3**). Evidence for stroke is much more limited, and out of 8 studies only 2 have found significant inverse association (253). IMT has been shown to be an independent predictor of CVD (12, 110), and thus our results in work I provided support for the protective role of flavonoids against CVD. Contrary, in work II the flavonoid intake was related with the incidence of ischemic stroke, but not with CVD mortality. Inconsistency in the findings on stroke may be partly explained by the differences in the study design or population studied; e.g. age, gender and types of stroke studied.

Despite optimism regarding beneficial effects of polyphenols against CVD, the high intake could merely be an overall marker of healthy lifestyle rather than a causative factor. Polyphenol

intake is strongly related with lifestyle habits. For example, the higher intake has been associated e.g. with less smoking, physical fitness as well as healthier diet; higher intake of e.g. vitamin C, vitamin E, carotenoids, folate, and fiber in our study and in other studies (197). Similar problem concerns the studies which have evaluated the role of foods sources of polyphenols such as tea. Therefore, the possibility that the protection at least partly results from other confounding lifestyle factors cannot be ruled out. In addition, most of the nutrients which are considered to be protective are often present in the same foods and therefore distinguishing the effects polyphenols from the other nutrients is difficult or even impossible.

6.4 Effects of polyphenol supplementation on serum lipids and oxidative stress

In three supplementation studies (works III-V), we found a little support for the antioxidant effects of polyphenols *in vivo* as only one marker of oxidative stress in one study showed beneficial change. In work V, oxidation susceptibility of serum was inhibited after consumption of flavan-3-ol rich phloem. However, no change was observed in the oxidation resistance of LDL+VLDL. Flavan-3-ol rich dark chocolate in work III did not have similar effect even though the type of flavonoids ingested was similar and the dose was several times higher than in the work V (62 mg per day vs. 418 mg per day). In addition, no effect was seen for coffee which contains high amount of phenolic acids. In work III, the production of conjugated dienes *in vivo* decreased, but this was probably due to fatty acids of chocolate, as an equal decrease was seen in all three study groups, and it was not related to the polyphenol content which varied between the chocolates.

The antioxidant effects of polyphenols in humans have been studied intensively since mid 1990s and these studies have mainly used tea, chocolate, cocoa, or red wine as a source of polyphenols (**Tables 4-6**). Majority of these studies favour the option that polyphenol supplementation increases the antioxidant capacity for a few hours (197). The effects on the markers of lipid peroxidation have been much more inconsistent (15). Studies which have included F_2-isoprostanes as a marker of lipid peroxidation *in vivo* have mainly not found polyphenol supplementation to have an effect. The inconsistency in the findings concerning the antioxidant effects in humans is not known, but may be related to the differences in the polyphenols supplemented (type and amounts), study subjects (age, health status, nutritional status, gender, etc.), study settings (duration, etc.) or methodology used to assess the effects. All in all, the results of polyphenols supplementation studies *in vivo* are inconclusive and it is not possible to draw final conclusions.

The main finding in work III was that cocoa increased the concentration of HDL cholesterol in a dose-dependent manner. In this study we did not study further which compounds are responsible

for the beneficial change in the HDL cholesterol. In addition to polyphenols, cocoa contains numerous of other compounds, such as minerals and methylxanthines. Even though polyphenols are not currently considered to have an effect on serum lipids, some supplementation studies (166, 185) have found cocoa to increase the HDL cholesterol as was in our study. The proportion of studies showing beneficial changes is, however, minority of the total number of studies. Further studies to verify the effects on HDL cholesterol are warranted.

7. SUMMARY

The results of this work are summarized as follows:

I The high intake of dietary flavonoids was associated with decreased carotid atherosclerosis measured as by common carotid artery intima-media thickness in middle-aged men.

II The high intake of dietary flavonoids was associated with a decreased risk of an ischemic stroke, while no association with cardiovascular disease mortality was found in middle-aged men.

III Flavonoids and other phenolic compounds do not seem to have measurable effect on oxidative stress in humans.

8. CONCLUSIONS

The results of this work suggest that the high intake of dietary flavonoids is associated with decreased carotid atherosclerosis and risk of ischemic stroke in eastern Finnish middle-aged men. However, we cannot fully exclude the possibility that a high flavonoid intake is an overall marker of healthy diet and lifestyle and not an independent preventive factor. The results of the supplementation studies do not support that the possible mechanism(s) by which flavonoids and other phenolic compounds may decrease the risk of CVD would be related with their antioxidant properties. More work is still needed to verify the effects of polyphenols on CVD and to identify the protective mechanism(s).

9. FUTURE DIRECTIONS

The role of dietary polyphenols in CVD is still unclear and further studies are still warranted. No single type of approach will provide a definite answer, and thus different types of studies should be carried out.

Epidemiological studies will provide useful information about the relation between the polyphenol intake and the risk of CVD. However, because of potential residual confounding, attention should be paid to the statistical adjustment. Furthermore, in order to evaluate the role of all commonly consumed polyphenols, efforts to update the databases should be continued.

The biomarkers of polyphenol intake have also been suggested for studying the effects of polyphenols on the risk of CVD in observational studies. However, because of extensive metabolism of these compounds, reliable evaluation of the total intake would require a great number of biomarkers. Therefore, relatively simple calculations about the intake of polyphenols will still be useful tool for epidemiological studies in the future.

In vitro and clinical studies are needed to verify the possible mechanisms of action. Antioxidant studies in humans have resulted in inconsistent findings and more studies should be done before conclusion about the effects can be drawn. In addition, developing markers of oxidative stress as well as establishing the role of these markers as predictors of CVD, should be continued. Studies assessing vasodilatory, antithrombotic and anti-inflammatory properties of flavonoids have provided promising results, but more evidence is needed.

Long-term randomized controlled trials are considered as a golden standard for establishing the effect of e.g. drugs. However, such approach has limitations in nutritional research. When studying the effects of long-term supplementation of polyphenols on CVD, sufficient contrast of exposure is difficult to arrange. A randomization of volunteers to follow diet rich in polyphenols (e.g. vegetables) for a long period of time (years, decades), while contrast group would follow diet low polyphenol diet, is impractical and unethical. Contrast could be possibly achieved with supplements, but adding them to the habitual diet would unlikely provide further benefit. Most nutrients have threshold behaviour i.e. the benefits are mainly achieved with a certain amount, but increasing the dose does not have any additional effect. Problem concerns especially nutritional studies, in which volunteers are likely to be more health conscious than average population.

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications referred to in the text by their Roman numerals I-V:

I Mursu J, Nurmi T, Tuomainen T-P, Ruusunen A, Salonen JT, Voutilainen S. The intake of flavonoids and carotid atherosclerosis: the Kuopio Ischaemic Heart Disease Risk Factor Study. British Journal of Nutrition 2007. In press.

II Mursu J, Voutilainen S, Nurmi T, Tuomainen T-P, Kurl S, Salonen JT. The high intake of flavonoids is associated with decreased risk of ischemic stroke in middle-aged Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study. Submitted.

III Mursu J, Voutilainen S, Nurmi T, Rissanen TH, Virtanen JK, Kaikkonen J, Nyyssönen K, Salonen JT. Dark chocolate consumption increases HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation in healthy humans. Free Radical Biology and Medicine 2004;37:1351-1359.

IV Mursu J, Voutilainen S, Nurmi T, Alfthan G, Virtanen JK, Rissanen TH, Happonen P, Nyyssönen K, Kaikkonen J, Salonen R, Salonen JT. The effects of coffee consumption on lipid peroxidation and plasma total homocysteine concentrations: a clinical trial. Free Radical Biology and Medicine 2005;38:527-534.

V Mursu J, Voutilainen S, Nurmi T, Helleranta M, Rissanen TH, Nurmi A, Kaikkonen J, Porkkala-Sarataho E, Nyyssönen K, Virtanen JK, Salonen R, Salonen JT. Polyphenol-rich phloem enhances the resistance of total serum lipids to oxidation in men. Journal of Agricultural and Food Chemistry 2005;53:3017-3022.

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