Carotenoid status in man: effects on biomarkers of eye, skin and cardiovascular health

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Stellingen

1. Daling van het carotenoïden gehalte in het bloed, binnen een normaal fysiologisch bereik en een periode van één jaar, heeft geen nadelige effecten op de gezondheid van het oog, de huid en hart en bloedvaten.
Dit proefschrift.

2. Een omgekeerde relatie tussen de dichtheid van het macula pigment en de lens, suggereert dat luteïne en zeaxanthine in het oog de veroudering van de lens kunnen vertragen.
O.a. dit proefschrift.

3. Het feit dat biomerkers vaak niet afdoende zijn gevalideerd, laat onverlet dat toepassing ervan waardevolle inzichten kan opleveren over mogelijke werkingsmechanismen van voeding in relatie tot gezondheid.

4. Het aanpassen van de smaak van groente en fruit kan bijdragen aan een gezonder en langer leven.
Mombaerts. Nature Genetics 2000;25:130-2.

5. Een staaroperatie is een voorteken voor de ontwikkeling van coronaire hartziekten.
Hu et al. American Journal of Epidemiology 2001;153:875-81.

6. 'Net zomin als er een veilige auto bestaat, bestaat veilige voeding. Veiligheid hangt immers nauw samen met hoe een product wordt gebruikt of geconsumeerd.'
C. Dutilh, De Volkskrant, 8 december 2001.

7. Door de ICT ontwikkelingen ter bevordering van het leggen en onderhouden van relaties raken veel mensen relationeel gezien in een isolement.

Stellingen behorend bij het proefschrift
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Abstract

Observational epidemiological studies have consistently shown that a diet rich in carotenoid-containing fruit and vegetables is associated with a reduced risk of chronic diseases. Because intervention studies with hard endpoints are time-consuming and costly, the use of biomarkers could be a promising approach. We studied the relation between serum carotenoid concentrations in a normal physiological range and biomarkers of eye, skin and cardiovascular health.

In a cross-sectional study among 376 subjects the associations between serum and adipose tissue lutein and macula pigment (MP) density in the eye were stronger in men than in women. MP density was inversely associated with lens density. Lens density was not related to serum and adipose tissue concentrations of lutein after adjustment for age. A modifying effect was observed on the association between serum carotenoids and minimal erythema dose, a marker of skin sensitivity to UV light. Several carotenoids were inversely associated with markers of inflammation markers and endothelial function.

In a 1-year randomized double blind placebo-controlled trial among 341 subjects, the effects of 0, 7, 10 and 17 g/d consumption of sucrose polyesters (SPE) on serum carotenoids and functional biomakers were studied. Lipid standardized carotenoid concentrations decreased by 13–33% in the group consuming 17 g/d SPE in comparison with the control group. Decreases of serum carotenoids in the 10 g/d and 7 g/d SPE groups were less. No negative effects were observed on markers of oxidation, eye health, cardiovascular health, and immune status. In a 4-week randomized single blind placebo-controlled trial among 47 subjects, the effect of 500 g/d fruit and vegetables + 200 mL fruit juice was studied in comparison with 100 g/d fruit and vegetables consumption on serum carotenoids and biomarkers of cardiovascular health. Serum carotenoids increased by 22–128%. Homocysteine concentrations decreased by 11%. No effect was observed on serum lipids, blood pressure and fibrinolysis/coagulation variables.

Overall, our studies used biomarkers that are indicative of biological processes currently thought to be important in disease etiology. But the predictive value has not been established. Therefore, our studies cannot be conclusive for disease risk. However, they seem to add proof to the hypothesis that serum carotenoid decreases or increases within normal physiological ranges in periods up to one year, have no or limited impact on biomarkers related to eye, skin and cardiovascular health.

Contents

Chapter 1

Introduction

The common denominator of the studies in this thesis is their focus on potential health effects of carotenoids in relation to eye, skin and cardiovascular health. Observational epidemiological studies have consistently shown that a diet rich in carotenoid-containing fruit and vegetables is associated with a reduced risk of chronic diseases, such as cardiovascular disease, some types of cancer, cataract and macular degeneration. Although the preventive mechanisms are not fully understood, many authorities now recommend an intake of more than 400 g/d of fruit and vegetables, and carotenoids are widely held to explain in part the beneficial effect of fruit and vegetables. Therefore, the introduction of products in which fat was replaced by non-digestible sucrose polyesters (SPE) met with scientific concern because SPE intake lowers plasma concentrations of carotenoids, probably associated with negative effects. This introduction should provide the reader with background information to comprehend the studies presented in this thesis. First, some information on carotenoids in man is given. Second, the state-of-the-art is summarized with regard to health effects of carotenoids on the eye, the skin and the cardiovascular system. Third, the concept of biomarkers is introduced and biomarkers of eye health, skin health and cardiovascular health are discussed with information on their relation to carotenoids, if available. Finally, the outline of the thesis and the research questions asked in this thesis are given.

Research on carotenoids

Up to now, β-carotene has been the most studied carotenoid, but the knowledge of other carotenoids is rapidly expanding (Van den Berg *et al.*, 2000). Earlier observational studies have shown quite consistently a reduced risk of cancer in individuals with a high intake of carotenoids or high blood carotenoid concentrations (van Poppel and Goldbohm, 1995). Based on these promising associations, intervention trials were executed to evaluate the cancer-protective effect of β-carotene supplementation. These large-scale studies of high doses of β-carotene (15–30 mg/day) have shown no effect (Hennekens *et al.*, 1996) or even an increased risk of lung cancer in smokers (ATBC Study Group, 1994; Omenn *et al.*, 1996). Only one study showed a protective effect of combined β-carotene, selenium and vitamin E supplementation (Blot *et al.*, 1993). Also, intervention trials did not confirm the protective effect of β-carotene on cardiovascular disease (Hennekens *et al.*, 1996). Recently, a high intake of lycopene was found to be

associated with a lower risk for prostate cancer (Giovannucci, 1999) and high lutein and zeaxanthin consumption with a lower age-related macular degeneration risk (Seddon *et al.*, 1994). Previous findings suggest that carotenoids other than β-carotene, probably at nutritionally relevant concentrations, may be important in preventing disease. Otherwise, carotenoid concentrations could reflect other components or processes in the body related to disease risk.

Recently, laboratory analyses allowed of measuring individual carotenoids in serum and other tissues, such as adipose tissue, prostate, skin and the eye (Stahl *et al.*, 2000a; Seddon *et al.*, 1994; Kardinaal *et al.*, 1995; Handelman *et al.*, 1988; Bone *et al.*, 1985). The main carotenoids found in blood are lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene in concentrations ranging from 0.05 to 0.40 μmol/L, while most other carotenoids are present in blood and tissues. Adipose tissue is readily accessible for biopsy and adipose tissue concentrations of carotenoids could also be used next to serum concentrations as a marker of carotenoid status (Kardinaal *et al.*, 1995).

Intervention studies have shown that blood carotenoid concentrations can be influenced by dietary intake. For example, fruit and vegetable consumption can increase serum carotenoid concentrations (Zino *et al.*, 1997; Yeum *et al.*, 1996). Absorption of dietary carotenoids is inhibited by non-absorbed lipophilic and lipid-like compounds. A few years ago, products in which fat was replaced by sucrose polyesters (SPE) were introduced in the US market. Consumption of these products with fat substitutes reduces the bioavailability of carotenoids and can lower serum carotenoid concentrations (Weststrate and van het Hof, 1995). More recently, products enriched with phytosterol and plant stanol esters capable of lowering LDL cholesterol were launched on the market. These products also diminish carotenoid absorption, albeit to a lesser extent (Weststrate and Meijer, 1998; Plat and Mensink, 2001). These changes in serum carotenoid concentrations may influence carotenoids concentrations in several tissues and, eventually, disease risk.

Health effects of carotenoids on the eye

The eyes contain many parts, all working together to process sensory information for vision. Light enters the eye at the corneal surface and passes through the lens to the posterior of the eye where the light is focused as an image on the retina (Taylor, 1999). The retina is a thin, transparent, light-sensitive neural tissue that originates

from the central nervous system during embryonic development. The retina is the actual organ of vision and contains the photoreceptors (cones and rods). These photoreceptors are responsible for the conversion of light into electric signals, which are transported by nerve cells to the brain. The macula is a structure near the centre of the retina that contains the fovea and a small pit, the fovea centralis. This specialized part of the retina contains a high concentration of photoreceptors and is responsible for vision thus allowing of such activities as reading (Taylor, 1999). This macula region in the retina is damaged by age-related macular degeneration (AMD), the leading cause of blindness in the Western world. One hypothesis is that oxidative stress could play a role in the pathogenesis of AMD. The retina is susceptible to oxidative stress due to high oxygen consumption, a high proportion of polyunsaturated fatty acids in the photoreceptors and exposure to visible light (Beatty *et al.*, 1999).

The carotenoids lutein, zeaxanthin and meso-zeaxanthin may play a critical role in the maintenance of normal vision because these polar compounds are the main carotenoids in the macula for shaping macular pigment (MP), while other, non-polar carotenoids are absent. The high concentration of these carotenoids is responsible for the yellow color, called macula lutea or 'yellow spot'. It has been hypothesized that these carotenoids reduce the risk of AMD by preventing damage to the retina by absorbing high-energy blue light or by virtue of their antioxidant activity (Landrum *et al.*, 1997a). Some observational epidemiological studies have shown a reduced risk of AMD in subjects with a higher intake of lutein and zeaxanthin or higher plasma concentrations of lutein and zeaxanthin (Seddon *et al.*, 1994; Eye Disease Case Control Study Group, 1993; Goldberg *et al.*, 1988). Other epidemiological studies have not shown significant reductions in risk of AMD with high serum concentrations or dietary intakes of lutein and zeaxanthin (Mares-Perlman *et al.*, 1995a; Sanders *et al.*, 1993; Mares-Perlman *et al.*, 2001). A recent large randomized placebo controlled trial showed that the intake of a supplement with both antioxidants (vitamin C, E and β-carotene) and zinc in comparison with placebo the development of advanced AMD statistically reduced. (AREDS, 2001a).

The primary function of the lens is to collect and focus light on the retina. The lens is a simple tissue made of epithelial cells and fiber cells. Epithelial cells on the anterior surface of the lens divide, transmigrate to the core of the lens and

differentiate into fiber cells. These fiber cells synthesize proteins, called crystallines, which fill the fiber cells, thus resulting in a clear lens (Taylor, 1999). As the lens ages, or with stress due to light exposure (McCarty and Taylor, 1999) or smoking (West, 1999), the proteins are photooxidatively damaged and aggregate, causing precipitations and an opaque lens, which interferes with vision and is involved in cataract (Taylor, 1999). Cataract is a major cause of visual disability leading eventually to blindness. Fortunately, surgical removal of the lens is successful in restoring adequate vision in most cases.

Because of the involvement of oxidative processes in the development of cataract, interest has focused on antioxidant compounds. Lens proteins are protected indirectly by antioxidant enzymes: superoxide dismutase, catalase and glutathione reductase/peroxidase. Direct protection is offered by antioxidants: glutathione (GSH), ascorbate (vitamin C), tocopherol (vitamin E) and carotenoids. The major lens carotenoids are lutein and zeaxanthin (Taylor, 1999).

In observational studies, consumption of foods rich in lutein and zeaxanthin or high serum concentrations of these carotenoids was associated with a lower relative risk of cataract (Chasan-Taber *et al.*, 1999; Brown *et al.*, 1999; Lyle *et al.*, 1999; Hankinson *et al.*, 1992; Jacques and Chylack, 1991). However, Mares-Perlman *et al.* (1995b) did not detect an altered risk for cataract among consumers of these specific carotenoids. Two prospective intervention studies on the effect of a high amount of antioxidants on cataract and vision-loss showed conflicting results. The AREDS study did not find an effect of vitamin C, E, β-carotene and zinc on the development or progression of cataract (AREDS, 2001b). The REACT (Chylack *et al.*, 2002) showed a small deceleration in the progression of cataract after supplementation with high amounts of vitamin C, E and β-carotene.

Health effects of carotenoids on the skin

The human skin is the largest organ of the body and acts as a protective covering of the body. Skin condition and functioning is affected by environmental factors, such as UV radiation, free radicals, toxic and allergenic compounds and mechanical damage, as well as by endogenous factors such as genetic predisposition, immune and hormone status and stress (Boelsma *et al.*, 2001).

The effects of UV light may be either beneficial or damaging. Sunlight is essential for the formation of vitamin D, and UV light exposure may be used successfully in

the treatment of some disease conditions like acne and eczema. Damaging effects induced by UV irradiation via light-dependent formation of reactive oxygen species (ROS) are the development of sunburn, aging of the skin and skin cancer. Protective effects of carotenoids in the skin have been shown in the treatment of a genetic disorder, erythropoietic protoporphyria. In this disease an enzyme defect causes the uptake of the photosensitizer protoporphytin in the skin, where it absorbs light and forms ROS. Treatment with large amounts of β-carotene leads to the accumulation of β-carotene in the skin which alleviates the symptoms (Mathews-Roth, 1993). Not only β-carotene can accumulate in the skin but also other carotenoids (Stahl *et al.*, 2000a; Peng *et al.*, 1995).
Observational data on the relation between carotenoids and risk of non-melanoma skin cancer are sparse and inconsistent. Some studies report an inverse relation between carotenoid concentrations and non-melanoma skin cancer (Wei *et al.*, 1994; Kune *et al.*, 1992) while others could not support the hypothesis that diets high in specific vitamins lower the risk of this disease (van Dam *et al.*, 2000). No effect was found in a large randomized trial of 12 years of supplementation with β-carotene on non-melanoma skin cancer (Frieling *et al.*, 2000).

Health effects of carotenoids on cardiovascular disease
Cardiovascular disease is the main cause of death in the Netherlands and other Western countries. The mechanisms leading to this disease are multifactorial and have not yet been fully unravelled. Several risk factors, including smoking, high homocysteine concentrations, high blood pressure and high serum cholesterol concentrations, adversely affect endothelial function.
Atherosclerosis is seen as an inflammatory disease (Ross, 1999). The vascular endothelium can be activated by a variety of inflammatory stimuli, including oxidized LDL, free radical species or high mechanical stress. The activated endothelium releases cytokines and up-regulates the expression of adhesion molecules. In normal conditions, this process is transient but when the inflammation continues, circulating monocytes are attracted to the endothelium by chemokines, bind adhesion molecules and transmigrate to the subendothelial space. Within the sub-endothelial space, they become macrophages, scavenge oxidized LDL and become foam cells. This contributes to the development of fatty streak in the early stage of atherosclerosis (Brown and Hu, 2001; Ross, 1999). Platelets can

adhere to the dysfunctional endothelium and then become activated, which results in loose thrombus formation, which is stabilized by the activation of the coagulation cascade. If the process of injury continues, intermediate and advanced, complicated lesions will be formed (Ross, 1999).

In general, epidemiological studies have found inverse associations between serum or adipose β-carotene concentrations and cardiovascular disease risk (Kardinaal *et al.*, 1993; Gey *et al.*, 1993; Street *et al.*, 1994). In contrast to the consistent results of the observational studies, intervention studies could not confirm the protective effects of carotenoids (Greenberg *et al.*, 1996; Omenn *et al.*, 1996; ATBC Study Group, 1994; Hennekens *et al.*, 1996). The discrepancy in results between observational studies and intervention studies could be explained by the fact that serum concentrations were confounded by one or more factors related to β-carotene concentrations and also predictive of cardiovascular disease, for example other constituents of fruit and vegetables (Kritchevsky, 1999).

Evidence that fruit and vegetables protect against cardiovascular disease is accumulating (Ness and Powles, 1997; Law and Morris, 1998; Liu *et al.*, 2000). Vegetables and fruit are rich sources of a variety of components, including vitamins, trace minerals, dietary fiber, folate and many other classes of bioactive compounds including carotenoids and flavonoids. These compounds could have different mechanisms of actions, for example modulation of cholesterol synthesis, modulation of detoxification enzymes, stimulation of the immune system, reduction of blood pressure and antioxidant effects (Lampe, 1999).

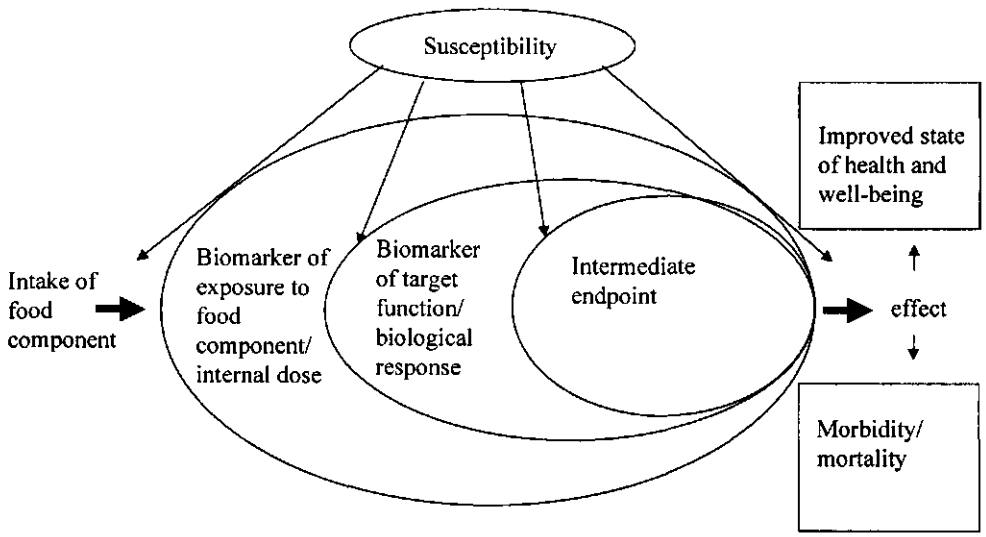
Biomarkers, or how to assess health effects of diet in human intervention studies

Formal proof for the positive or negative effects of nutritional compounds requires clinical trials with hard end-points, such as morbidity or death. Such studies are indispensable but time-consuming and costly. A promising approach to strengthen epidemiological studies seems to be the use of biomarkers that can be used as predictors of the actual effect of diet on disease.

The European Commission Concerted Action on Functional Food Science (FUFOSE), which was co-ordinated by the International Life Science Institute (ILSI), developed a consensus document on the identification and validation of relevant markers that can predict potential benefits or risks related to a target

function in the body. In the causal pathway between intake and effect, one might distinguish biomarkers by their relation with different processes, namely biomarkers of exposure or internal dose, biomarkers of target function/biological response and intermediate end-points, the latter two designated as biomarkers of enhanced function and biomarkers of reduced risk (Diplock *et al.*, 1999). Serum concentrations of carotenoids or vitamins (marker of exposure/internal dose) can be studied in relation to oxidation of LDL (biomarker of biological response) or narrowing of the carotid artery wall (intermediate end-point as evidence for cardiovascular disease) in subjects with different genotypes (susceptibility marker).

Figure 1 Classification of markers (modified from Diplock *et al.*, 1999)



In the evaluation of suitability of biomarkers, such properties as relevance, validity, reproducibility and feasibility must be studied (Diplock *et al.*, 1999). Firstly, the measured biomarker must have biological relevance for objective of the study; markers must be clearly linked to the phenomena involved in the biological processes being studied. Biomarkers must be validated analytically in standard quality control procedures. Biomarkers must also be reproducible, i.e. consistent outcomes within observers, among observers and among different centres. Validity of biomarkers of exposure is established when a change in intake predicts a change

in the biomarkers of exposure, while the validity of biomarkers of enhanced function or reduced disease risk is established when a change in these biomarkers predicts a change in disease risk. The feasibility of biomarkers in human intervention studies is dependent on analytical considerations (e.g. sample collection, storage and transport conditions, time required for laboratory analyses) and ethical considerations (e.g. easily accessible material, minimally invasive), and the biomarker must be potentially modifiable within the time frame of the study.

Biomarkers of eye health

Macular pigment (MP) in the eye could be measured by spectral reflectance analysis (van de Kraats *et al.*, 1996). Spectral reflectance analysis is a reliable method that could be used in a naive target population, with a within subject variation of 17% (Berendschot *et al.*, 2000). MP density can also be measured by heterochromatic flicker photometry (Hammond *et al.*, 1997a), MP maps from scanning laser ophthalmoscopy (Berendschot *et al.*, 2000), the resonance Raman scattering method (Bernstein *et al.*, 1998, 2001) and autofluorescence spectrometry (Delori *et al.*, 2001). The estimates of MP density values by these methods are highly correlated mutually (Delori *et al.*, 2001, Berendschot *et al.*, 2000).

Results of previous studies show that MP can be influenced by consumption of green leafy vegetables and corn (Hammond *et al.*, 1997a; Johnson *et al.*, 2000) and the use of lutein supplements within several weeks (Berendschot *et al.*, 2000; Landrum *et al.*, 1997b). The relation between MP density and AMD is not clear yet. Bone *et al.* (2001) showed that donor eyes from subjects with AMD had 30% lower MP density values than donor eyes of subjects without AMD. This supports the hypothesis that MP density could protect against AMD. In contrast, Berendschot *et al.* (in press) have found that there was no difference in MP density between healthy subjects and patients with different stages of AMD. In addition, it is not known if a change in MP density can accurately predict a change in risk of AMD.

Spectral reflectance analyses has been shown to yield reliable estimates of lens density (van de Kraats *et al.*, 1996). There is a strong relation between lens aging and cataract, the latter being the end stage of deterioration of the aging lens (Sample *et al.*, 1988; Bron *et al.*, 2000). Although these studies show a strong positive association between lens density and age, there are substantial individual

differences irrespective of age. Other risk factors of cataract or nutrition could play a role in these individual differences. MP density could be used as a marker of lutein and zeaxanthin in the eye. It has been shown that MP density correlates inversely with lens density (Hammond *et al.*, 1997b).

Biomarker of skin health

Sunburn is a well-known acute effect of excessive UV exposure. Low-dose or brief exposure to UV irradiation is tolerated by the skin without noticeable or clinically relevant changes. Only after reaching a certain threshold, tissue damage results in a delayed and prolonged vasodilation allowing passage of lymphocytes and macrophages into the tissue and inducing an inflammatory response, which is clinically visible as erythema (Boelsma *et al.*, 2001).

A frequently used measure for the skin's sensitivity to UV light is the minimal erythema dose (MED). One MED is the minimal amount of energy required to induce visible erythema, which can be defined as a uniform, clearly demarcated or just perceptible redness at 16–24 h after UV exposure (Boelsma *et al.*, 2001). Thus, a low MED indicates a high sensitivity to UV irradiation. The coefficient of variation is <12.5% (A. Vink, personal communication).

Carotenoid supplements have been shown to provide moderate protection against sunburn as measured by erythema (Stahl *et al.*, 2000b, Lee *et al.*, 2000). Just one study with tomato paste (Stahl *et al.*, 2001) showed a positive relation between dietary intake of lycopene and erythema, which suggests that lycopene decreases the sensitivity of the skin to UV light. Sun exposure is the main established risk factor for non-melanoma skin cancer (Armstrong and Kricker, 2001), but the relation between skin sensitivity to UV light as measured by MED and the resulting risk of skin cancer is not established.

Biomarkers of cardiovascular health

Biomarkers of cardiovascular disease (CVD) can be classified as risk factors, such as blood pressure, cholesterol and homocysteine, and as manifestations of altered function, such as impaired endothelial function or intima-media thickness of the carotid artery wall. Impaired endothelial function can be measured by enhanced endothelial activation and impaired endothelium-dependent vasodilation as markers of altered function. Endothelial activation is reflected in increased plasma

concentrations of soluble adhesion molecules, including vascular cell adhesion molecules (VCAM-1), intercellular adhesion molecules (sICAM-1), E-selectins and P-selectins. These molecules are involved in leukocyte recruitment and platelet adhesion during thrombosis and inflammation (Brown and Hu, 2001). Concentrations of these adhesion molecules are elevated in patients with ischemic heart disease (Morisaki *et al.*, 1997) and coronary heart disease (Hwang *et al.*, 1997) and could predict future risk of myocardial infarction (Ridker *et al.*, 1998) and coronary artery disease (Blankenberg *et al.*, 2001). Endothelium-dependent vasodilation can be measured by a non-invasive ultrasound technique, which measures the increase in arterial diameter induced by a local increase in arterial blood flow (de Roos *et al.*, 2001). Numerous studies have confirmed impaired vasodilation in patients with CVD (Celermajer *et al.*, 1992; Cox *et al.*, 1989; Zeiher *et al.*, 1991). Several studies have consistently shown that inflammation markers, such as C-reactive protein (CRP), fibrinogen and amyloid A, can predict cardiovascular disease (Danesh *et al.*, 1998; Ridker *et al.*, 2000). Arterial thrombosis can be measured by coagulation and fibrinolysis markers, but their causal role in relation to disease has to be established.

Carotenoids might play a role in the protection against the initiation of CVD by virtue of their antioxidant function which may protect against LDL-oxidation or through other mechanisms, such as anti-inflammatory processes. Some intervention studies show a preventive effect of several carotenoids on LDL oxidation (Agarwall and Rao, 1998; Uprichard *et al.*, 2000; Bub *et al.*, 2000). Inflammation processes have to be taken into account when studying the relation between carotenoids and CVD, as suggested by several groups (Kritchevsky, 1999; Kritchevsky *et al.*, 2000; Erlinger *et al.*, 2001). Previous observational studies have shown negative associations between serum carotenoids and inflammation markers, such as CRP and leucocytes (Kritchevsky *et al.*, 2000; Erlinger *et al.*, 2001). In an intervention study in patients with diabetes type II no effect was seen on markers of inflammation after tomato juice consumption (Uprichard *et al.*, 2000).

Other components in fruit and vegetables

Many components of fruit and vegetables could have an influence on the mechanisms underlying CVD. Results of most published studies support a role for vitamin C and vitamin E in preserving endothelium-dependent vasodilation mediated, at least in part, by their antioxidant activity (Brown and Hu, 2001). Folic acid can have a beneficial effect on the vascular endothelium by reducing plasma homocysteine concentrations or through other mechanisms by reducing oxidative stress (Wilmink *et al.*, 2000). In a four-week controlled, parallel-design dietary intervention trial it was shown that dietary folate (560 μg) from vegetables and citrus fruit could decrease homocysteine concentrations by 2.0 μmol/L (95% CI 1.0–3.0) (Brouwer *et al.*, 1999). In a large randomized controlled trial it was shown that a diet rich in fruit and vegetables (ca. 8.5 servings per day) could reduce systolic blood pressure by 2.8 mmHg ($P < 0.0001$) and diastolic blood pressure by 1.1 mmHg ($P = 0.07$) after 8 weeks (Appel *et al.*, 1997). A diet consisting of both fruit and vegetables and low-fat dairy products and with reduced saturated and total fat concentrations ('combination diet') reduced blood pressure even more. As a secondary outcome of this trial, serum lipids were not significantly reduced by the fruit and vegetable diet, although the 'combination diet' reduced total cholesterol, LDL cholesterol and HDL cholesterol by 7.3%, 9.0% and 7.5%, respectively (all $P < 0.0001$) (Obarzanek *et al.*, 2001). In another intervention trial a recommendation to increase fruit and vegetable consumption alone produced no change in serum lipids despite significant increases in serum carotenoid and vitamin C concentrations (Zino *et al.*, 1997). Human studies that relied on other biomarkers possibly related to chronic disease risk have been reviewed by Lampe (1999).

Outline of this thesis

As mentioned above, carotenoids are potential beneficial components in fruit and vegetables in relation to chronic diseases given the results of observational epidemiological studies. Intervention trials have shown that high-dose β-carotene supplementation has no protective effect or even increases disease risk. Therefore, it seems advisable to study the effects of those carotenoids which are most abundant in the diet at nutritionally relevant concentrations with regard to disease risk, in particular diseases in which carotenoids are found in the target tissue of the disease, for example the eye as target tissue in age-related macular degeneration.

Carotenoids are not the only potential preventive compounds in fruit and vegetables against chronic diseases, such as cardiovascular disease. Therefore, it seems also advisable to study the effect of fruit and vegetables as a whole at nutritionally relevant consumption concentrations on disease risk. Because intervention studies with hard end-points are time-consuming and costly, the use of biomarkers as predictors of the actual effect of diet on disease could be a promising approach.

The objective of the studies reported in this thesis was to evaluate the effects of carotenoid status in normal physiological ranges in man on biomarkers of eye, skin and cardiovascular health. This thesis describes the results of an observational study and two human intervention studies. The results presented for the observational study were obtained from the baseline measurements of an intervention study. In this first intervention study, the effects of SPE consumption was studied on biomarkers of eye, skin and cardiovascular health. In a second intervention study, the effects of a high but realistic intake of fruit and vegetables were assessed on biomarkers of cardiovascular health.

In the observational study the following research questions were addressed:

1. What is the quantitative relation of serum and adipose tissue carotenoid concentrations in a normal physiological range with biomarkers of eye health in human volunteers? Macular pigment (MP) density (Chapter 2) and lens density (Chapter 3) were measured as biomarkers of eye health. Chapter 3 also evaluates the relation between MP density and lens density.

2. What is the quantitative relation of serum carotenoid concentrations in a normal physiological range with sensitivity of the skin to UV light in human volunteers? Minimal erythema dose (MED) was used as a biomarker of skin health. In addition, we evaluated the associations between phenotypic correlates, such as hair, skin and eye color, and MED (Chapter 4).

3. What is the quantitative relation of serum carotenoid concentrations in a normal physiological range with biomarkers of endothelial function and inflammation in human volunteers? Results on intercellular adhesion molecule (sICAM-1), flow-

mediated vasodilation (FMD), C-reactive protein (CRP), fibrinogen (Fbg) and leucocytes are reported in Chapter 5.

In the intervention studies the following research questions were addressed:
4. What is the effect of a long-term intake of sucrose polyesters (SPE) as part of a normal dietary pattern on serum carotenoids and fat-soluble vitamins, markers of oxidative damage and functional markers of eye and cardiovascular health and immune status?
Studies on the long-term health effects of decreased carotenoid concentrations or effects of SPE are lacking. Therefore, we conducted a 1-year randomized, double blind, placebo-controlled parallel human intervention trial to investigate the possible effects of a long-term decrease in carotenoid concentrations, caused by SPE, on biomarkers of oxidation, eye, cardiovascular health and immune status (Chapter 6).

5. What is the effect of an increased fruit and vegetable consumption on serum carotenoids, vitamins and markers of cardiovascular risk profile?
Besides carotenoids, fruit and vegetables contain other potential beneficial compounds protecting against the risk of chronic diseases. It is thought that this combination of compounds, provided by consumption of a variety of fruit and vegetables, is responsible for the beneficial effects of fruit and vegetables. We performed a 4-week randomized placebo-controlled parallel human intervention trial to investigate the effect of an increased fruit and vegetable consumption on serum carotenoids, vitamins and markers of cardiovascular health (Chapters 7 and 8).

In the general discussion (Chapter 9), main findings are summarized and discussed, that are related to carotenoid status in a normal physiological range on biomarkers of enhanced function or reduced risk. Secondly, the predictive value of the biomarkers used in our studies is discussed.

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Chapter 2

Macular pigment density in relation to serum and adipose tissue concentrations of lutein and serum concentrations of zeaxanthin

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Abstract

Background: Macular pigment (MP), concentrated in the central area of the retina, contains the carotenoids lutein and zeaxanthin. A low MP density could be a risk factor for age-related macular degeneration (AMD). Little information is available regarding MP density in relation to serum lutein and zeaxanthin and adipose lutein in a general population.

Objective: To investigate the associations between serum lutein and zeaxanthin, adipose lutein and MP density, taking into account potential confounders in a population.

Design: 376 volunteers aged 18–75 years were recruited. In a cross-sectional design, serum (n = 376) and adipose tissue (n = 187) were analyzed for carotenoids and MP density was measured by spectral fundus reflectance.

Results: Mean MP density in the total study group was 0.33 ± 0.15. MP density was 13% higher in men than in women ($P < 0.05$). Serum and blood concentrations were significantly higher in women for α-tocopherol, vitamin C and all carotenoids except for lycopene. Adipose lutein concentrations were also significantly higher in women than in men. Regression models showed a positive significant association between serum lutein, serum zeaxanthin, adipose lutein concentrations and MP density in men after adjustment for age, but no relation in women. In men, serum lutein was still significantly associated with MP density after adjustment for age, total cholesterol, BMI and smoking.

Conclusion: The associations between MP density and serum lutein, serum zeaxanthin and adipose lutein concentrations are stronger in men than in women.

Introduction

Age-related macular degeneration (AMD) is the main cause of visual impairment in Western countries and affects the central region of the retina with the highest degree of visual acuity, the macula lutea. Macular pigment (MP) possibly acts as a blue light filter to protect the macular region against photo-oxidation by light (Landrum *et al.*, 1997a). In addition, MP is capable of scavenging free radicals (Khachik *et al.*, 1997). The carotenoids lutein and zeaxanthin are the predominant pigments in this area (Snodderly, 1995). Some observational epidemiological studies have shown a reduced risk of AMD in subjects with a higher intake of lutein and zeaxanthin or higher plasma concentrations of lutein and zeaxanthin (Seddon *et al.*, 1994; Eye Disease Case-Control Study Group, 1993; Goldberg *et al.*, 1988). Other epidemiological studies have not shown significant reductions in risk of AMD with high serum concentrations or dietary intakes of lutein and zeaxanthin (Mares-Perlman *et al.*, 1995; Sanders *et al.*, 1993; VandenLangenberg *et al.*, 1998; Mares-Perlman *et al.*, 2001). MP density was associated with preservation of visual sensitivity in another study (Hammond *et al.*, 1998). The possibility that MP is protective against AMD is likely but further research is necessary (Beatty *et al.*, 1999).

If it is demonstrated that MP density has a protective effect on AMD, it is important to understand the influence of several factors on MP density and on the relationship between MP density and serum concentrations of lutein and zeaxanthin, a possible biochemical indicator of intake of carotenoids (Hunter, 1990).

In previous studies, MP density was positively related to dietary intake and serum concentrations of lutein and zeaxanthin (Hammond *et al.*, 1996a; Bone *et al.*, 2000). A dietary intervention study showed that an increase in intake of lutein and zeaxanthin from foods could increase MP density (Johnson *et al.*, 2000). Two other studies reported a change in MP density through lutein supplements (Landrum *et al.*, 1997b; Berendschot *et al.*, 2000). In one study some subjects failed to show a change in MP density after increasing dietary intake of lutein and zeaxanthin (Hammond *et al.*, 1997).

Previous studies have shown an influence of several factors on the relationship between MP density and serum concentrations of lutein and zeaxanthin, for example sex and smoking (Hammond *et al.*, 1996a; Hammond *et al.*, 1996b). In

addition, in men, a significant positive correlation was found between MP density and adipose lutein concentration (Johnson *et al.*, 2000), another possible biochemical indicator of carotenoid intake (Hunter, 1990). In contrast, they reported a negative correlation between adipose lutein and MP density in women. So far, these associations were only evaluated in small study samples. We determined the relation between serum lutein, serum zeaxanthin and MP density in 376 volunteers, adjusting for possible confounding factors. The relation between adipose tissue lutein concentrations and MP density was determined in 187 volunteers, adjusted for possible confounding factors.

Subjects and methods

Subjects

The 380 subjects in our study were recruited from the pool of volunteers of TNO Nutrition and Food Research and through advertizing in local and regional newspapers and on television. The cross-sectional data analyzed here were gathered during the baseline measurements of an intervention study designed to test an unrelated hypothesis. Advertizing aimed at recruiting people with a general interest in participating in nutrition studies. Respondents who expressed potential interest ($n = \pm 6900$) received a form with the in- and exclusion criteria of the study and a questionnaire requesting data on daily fruit and vegetable consumption and lifestyle factors. This questionnaire was designed for ranking and selection of subjects according to their intake and not for quantitative measurements. It was a simple questionnaire consisting of questions about the usual fruit and vegetable intake, including fruit juices over the last month. This questionnaire contained 8 items about fruit and vegetable intake, including fruit juices in days/week. In addition, 8 questions were included about the portion size, 4 questions about food item specification and 1 question about vitamin and/or mineral supplements.

The major inclusion criterion was an age of 18–75 years. The main exclusion criteria were pregnancy and/or lactation or wishing to become pregnant, serum cholesterol > 7.5 and/or triacylglycerol > 2.3 mmol/L if not under stabilized hypercholesterolemia/ hyperlipidemia treatment, and anticoagulant therapy, and vegetarians and vegans.

A total of 2734 questionnaires were returned. Respondents in the highest quintile of fruit and vegetable consumption and those in the lowest tertile (calculated in the

total group) were selected. In the lowest stratum vitamin supplement users were excluded (n = 127). Finally, we invited 547 respondents of the highest quintile and 775 respondent of the lowest tertile for an oral briefing. At the oral briefing volunteers were informed about the measurements, blood sampling and macular pigment density measurement, and about an extra measurement in a subgroup, a fat biopsy. The volunteers were told that a fat biopsy could be painful for people with less adipose tissue on the buttocks. They had the opportunity to refuse sampling. After the oral briefing 589 volunteers were interested in participation and were screened. Informed consent was obtained from all subjects.

During the screening, 54 volunteers were excluded because of the exclusion criteria for serum cholesterol and/or triacylglycerol. A number of 122 volunteers withdrew during the screening. A total of 413 volunteers were allocated to an entry number. Before the start of the study, 33 subjects withdrew from the study.

At day one, 380 volunteers (179 men, 201 women) entered the study. This group consisted of 216 subjects from the low fruit and vegetable intake group and 164 subjects from the high fruit and vegetable intake group. We randomly selected volunteers from whom we would obtain an adipose tissue sample taking sex and age into account. If initially selected volunteers refused this measurement, other volunteers were randomly selected.

The study was performed according to ICH (International Conference on Harmonization of Technical Requirements of Registration of Pharmaceuticals for Human Use) guidelines for good clinical practice, and was approved by an external Medical Ethical Committee. The study was conducted at the Department of Nutritional Physiology of TNO Nutrition and Food Research, Zeist, and the measurements were performed from June 29 to September 16, 1998.

The final study group (95% whites) comprised 177 men aged 42 ± 15 years and 199 women (pre- and postmenopausal) aged 41 ± 13 years. Of these, 120 (68%) and 144 (72%) were non-smokers, respectively (see Table 1 and 2). Of this study group we obtained an adipose tissue sample from 89 men aged 43 ± 15 years and 98 women aged 42 ± 14 years. Of these volunteers, 63 (71%) and 68 (69%) were non-smokers, respectively.

Blood and adipose tissue sampling

Blood samples from subjects were obtained between 800 and 930h after an overnight fast. For the analysis of carotenoids and α-tocopherol blood was collected in tubes containing clot activator and gel (Becton Dickinson, Vacutainer systems). These tubes were immediately stored in a closed box, to avoid breakdown of carotenoids by UV light. Tubes were centrifuged within 15–30 min after collection at ca. 2000 × *g* for 10 min at ca. 4°C to obtain serum. After centrifugation serum was removed and stored at ca. –80°C. All serum handling before storage was done in subdued light.

For the analysis of vitamin C, blood was collected in tubes containing lithium heparin (Becton Dickinson, Vacutainer systems). A 0.5 mL aliquot of blood was added to 2 mL metaphosphoric acid (50 g/L; Mallinckrodt Baker, Deventer, Netherlands) before freezing to preserve the vitamin C concentration during storage. This mixture was stored at ca. –80°C.

For the analysis of total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol blood was collected in tubes containing clot activator and gel (Becton Dickinson, Vacutainer systems). Tubes were centrifuged within 15–30 min after collection at ca. 2000 × *g* for 10 min at ca. 4°C to obtain serum. After centrifugation serum was removed and stored at ca. –20°C.

Subcutaneous adipose tissue (approximately 50 mg) was obtained by needle biopsy from the lateral buttock (Beynen and Katan, 1985), using a 16-gauge needle attached to a plastic container in which the tissue was collected by connecting a vacuum tube. Samples were kept in the plastic container, immediately placed on dry ice and stored at ca. –80°C.

Chemical analyses

Serum samples

At the day of analysis serum samples were thawed and mixed well. Samples were deproteinized by adding 500 µL sample to 500 µL ethanol (Nedalco, Bergen op Zoom, Netherlands) containing tocol (3,4-Dihydro-2-methyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-ol, Matreya, Pleasant Gap, USA) as internal standard. After vortex mixing the samples were allowed to stand for 15 min at room temperature to complete precipitation of proteins. Subsequently, 1.0 mL *n*-hexane (Merck, Darmstadt, Germany) was added and vortexed for 4 min. After

centrifugation (2000 × *g*, 10 min, 4°C), 0.5 mL of the upper hexane layer was evaporated to dryness. The residue was dissolved in 0.5 mL of a mixture of methanol (Rathburn Chemicals, Walkerburn, UK), acetonitrile (Biosolve, Valkenswaard, Netherlands) and dichloromethane (Mallinckrodt Baker). A 50 µL portion was injected on the HPLC system. Quality of the measurements was assessed by analyzing plasma quality control samples in each batch. The coefficient of variation for the analysis of lutein, zeaxanthin, β-cryptoxanthin, all-*trans* lycopene, α-carotene, β-carotene and α-tocopherol were 3.7, 11.5, 8.3, 6.2, 14.9, 5.8 and 2.8%, respectively (at mean concentrations of 0.16, 0.03, 0.11, 0.31, 0.05, 0.25 and 20.9 µmol/L in the quality control samples, respectively). The HPLC system consisted of the following equipment: a Waters 2690 solvent delivery/injection system (Waters, Etten-Leur, Netherlands), a Waters 996 diode array and a Jasco 920 fluorescence detector (Jasco Benelux, Maarssen, Netherlands). System control and data acquisition/processing was performed by Millennium[32] chromatography manager version 3.05 (Waters).
Separation was obtained by two C18 reversed-phase columns (250 mm × 4.6 mm, 5 µm) in series, thermostatically controlled at 30°C. The samples were eluted using a gradient consisting of methanol, acetonitrile, 2-propanol (Mallinckrodt Baker) and milliQ® (Millipore, Etten-Leur, Netherlands) at a flow rate of 1.5 mL/min. This HPLC system allows simultaneous detection of retinoids, tocopherols and carotenoids as well as their isomers (Figure 1). Additionally, the diode array data provide spectral information for confirmation of the identity of the analytes.

Fat biopsies

Fat biopsies of about 50 mg were transferred to glass tubes and weighed accurately. After addition of 0.4 mL 10% sodium ascorbate solution (Sigma-Aldrich Chemie, Steinheim, Germany) and 2.0 mL 1.5 mM ethanolic KOH solution (Merck) the samples were saponified for 30 min at 80°C. Subsequently, the samples were cooled down to room temperature, 2.0 mL di-isopropyl ether (Merck) was added and the samples were mixed vigorously. Of this extract 2.5 mL was vortexed with 2.5 mL water for 4 min. The upper di-isopropyl ether layer was isolated and evaporated to dryness. The residue was dissolved in 0.1–0.2 mL of a mixture of methanol, acetonitrile and dichloromethane. A 50 µL portion was injected on the HPLC system, the same system as for the serum samples. In each series quality

control samples consisting of a mixture of butter and olive oil were analyzed to check for reproducibility. The concentration of the quality control sample was 0.39 μmol/kg lutein with a coefficient of variation of 6.8%.

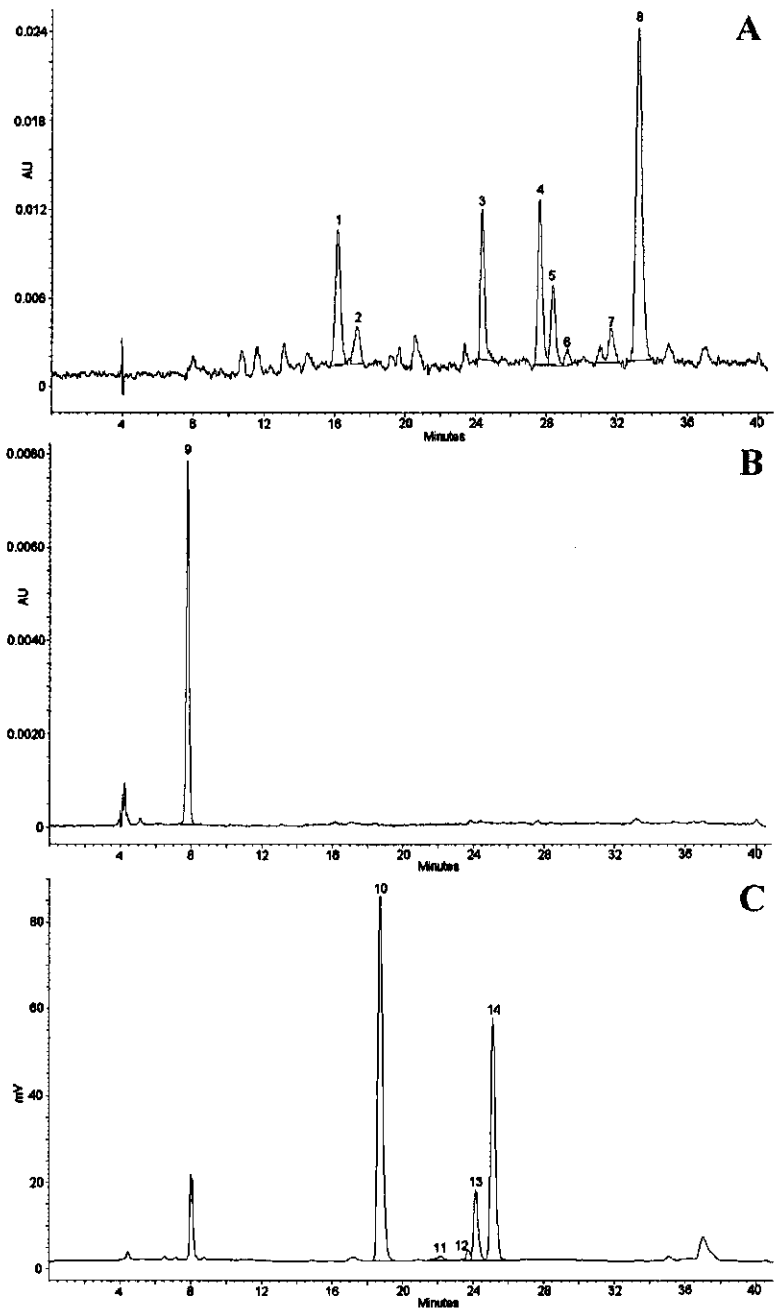
Blood samples

At the day of analysis blood samples were thawed and mixed well. Vitamin C was extracted from whole blood using metaphosphoric acid (Mallinckrodt Baker). Samples were allowed to stand for 20 min at 4°C to precipitate the protein and oxidize ascorbic acid to dehydroascorbid acid. After centrifugation samples were subsequently derivatized with 1,2-diaminobenzene (Merck) to quinoxline and injected on the HPLC system. In each series blood quality control samples were analyzed to check for reproducibility. The reproducibility of the assay was 10%. HPLC analysis was performed on an isocratic reversed-phase system consisting of a Gilson 234 injector (Meyvis, Bergen op Zoom, Netherlands), a Gynkotek model 300 pump (Separations, Hendrik-Ido-Ambacht, Netherlands), a C18 column (125 mm × 4.6 mm, 5 μm) and a Jasco 920 fluorescence detector. Mobile flow rate was 1.1 mL/min and consisted of methanol and buffer pH 7.8. Data acquisition and processing were performed by Turbochrom version 6.1.2.0.1:D19 (PerkinElmer Instruments, Nieuwerkerk a/d IJssel, Netherlands).

Macular pigment density measurement

MP density was measured in the right eye, if possible. The subjects' pupils were dilated with tropicamide 0.5% (Chauvin, Brussels, Belgium). Spectral fundus reflectance was measured with the Utrecht Retinal Densitometer (Van de Kraats *et al.*, 1996). Briefly, a rotating wheel (14 revolutions per second) offered a sequence of 14 interference filters in the range 430 to 740 nm to enable quasi-simultaneous measurement of the reflectance across the visual spectrum. Light reflected from the fundus was measured in a detection field of 1.5°, concentric within the illumination field of 1.8°. To obtain an estimate of the mean MP density in this area, spectral fundus reflectance was measured in two conditions: (1) with the instrument's entry and exit pupils aligned to the peak of the Stiles-Crawford (SC) function, and (2) 2 mm temporal to the SC peak. A detailed optical model of foveal reflection was used to arrive at individual estimates of parameters as equivalent thickness of blood

Figure 1 Chromatogram of carotenoid, tocopherol and retinol analyses[1]



[1]A. Carotenoids (UV at 450 nm)
1: lutein
2: zeaxanthin
3: β-cryptoxanthin
4: all-*trans* lycopene
5+6: isomers of lycopene
7: α-carotene
8: β-carotene
B.
9: all-trans retinol (UV at 325 nm)

C. Tocopherols (fluorescence; excitation 295 nm, emission 320 nm)
10: rac-tocol
11: δ-tocopherol
12: β-tocopherol
13: γ-tocopherol
14: α-tocopherol

layer and densities of MP, lens and melanin (Van de Kraats *et al.*, 1996). In short, the incoming light was assumed to reflect at the inner limiting membrane, the disks in the outer segments of photoreceptors and the sclera. Using known spectral characteristics of the different absorbers within the eye (lens, MP, melanin, blood), the densities of the pigments and percent reflectance at the interfaces were optimized, to fit the measured data at all wavelengths. A previous study using the same method showed a within subject variation coefficient of 17% (Berendschot *et al.*, 2000).

Measurement of potential confounders and baseline characteristics

Iris color and smoking status were determined because of their previously documented associations with MP density (Hammond *et al.*, 1996b; Hammond *et al.*, 1996c). Iris color was graded by a set of four standard photographs, providing a five-grade classification system (Seddon *et al.*, 1990). Blue and gray irises were classified as grade 1 or 2. These grades were distinguished by assessing the proportion of total iris area with brown or yellow pigment. A green iris was assigned grade 2 or 3. Similarly, these grades depended on the extent of brown or yellow pigment in the iris. A predominantly brown iris was assigned grade 3, 4 or 5, depending on the intensity of the yellow or brown pigment (Seddon *et al.*, 1990). Smoking status was obtained by a questionnaire; subjects were classified into 6 categories ranging from non-smoking (category 0) to more than 15 cigarettes/day (category 5).

Serum triacylglycerols were analyzed by enzymatic hydrolysis with subsequent enzymatic determination of the liberated glycerol by colorimetry (commercial available kit; Boehringer, Mannheim, Germany). Total cholesterol was analyzed by enzymatic conversion to a stable chromogen, which can easily be detected by colorimetry (Boehringer, Mannheim, Germany). HDL cholesterol was analyzed by precipitation with polyethylene glycol, centrifugation and enzymatic detection by colorimetry (Boehringer, Mannheim, Germany) and LDL cholesterol was calculated using the Friedewald formula (Friedewald *et al.*, 1972).

Further, age was determined, body weight and height measured and body mass index (BMI) calculated. Body weight was assessed by weighing the subject wearing indoor clothing, without shoes, wallet and keys. Height was measured

without shoes. Body mass index (BMI) was calculated with the formula body weight (kg)/ body height squared (m^2).

Statistical analyses

The SAS statistical software package SAS/STAT (Version 6, SAS Institute, Cary, NC, USA) was used for data analysis. T-tests were used to evaluate the differences in serum carotenoids, vitamins, MP density and continuous baseline characteristics between men and women, smokers and non-smokers and high and low fruit and vegetable intake. ANOVA was used to evaluate differences in MP density between different iris color categories. If we found significant differences between categories, the Scheffé test was used for multiple comparisons. Pearson correlation coefficients were calculated between baseline characteristics and both serum lutein concentrations and MP density. Correlation coefficients, adjusted for total cholesterol were calculated between serum lutein, MP density and other carotenoids and vitamins. If variables were not normally distributed, variables were log-transformed. Linear regression (GLM procedure) was used to evaluate the association between MP density and serum lutein and zeaxanthin concentrations and adipose lutein concentrations.

Firstly, two linear models with the independent variables serum lutein or zeaxanthin, adjusted for age were fitted (Model 1a-1b). Then, a model with both serum lutein and zeaxanthin, adjusted for age, was performed (Model 1c). This was our basic model. After inclusion of the sex variable and the interaction term lutein × sex or zeaxanthin × sex in the models, these interaction terms were significant ($P < 0.05$). This resulted in final general linear models for men and women separately. Variables that could influence the association between serum lutein and macular pigment density were investigated as potential confounders: smoking status and BMI. The models were adjusted for each potential confounder separately. The regression coefficient of serum lutein after adjustment for each potential confounder separately was compared with the regression coefficient of serum lutein in the basic models for men and women. If the change in the magnitude of the regression coefficient was more than 10%, the confounder was added to the model. Total cholesterol was also included in the model, because serum carotenoids were related to serum lipid concentrations. We also corrected for other carotenoids and vitamins to evaluate whether they were confounders.

Mean serum lutein concentrations were calculated across quartiles of lutein divided by total cholesterol in men and women. Because of the heterogeneity of the female group (pre- and postmenopausal), a regression analysis to evaluate the association between serum lutein and MP density was performed among women older and younger than 50 years. Previous procedures were also used to analyze the association between adipose tissue lutein concentration and MP density. If the *P*-value was less than 0.05, the regression coefficients of the variables were considered as significant ($P < 0.05$). Regression diagnostics were used to examine the residuals and influential points and test the model assumptions.

Two volunteers squinted too much during the MP density measurement. Further, one measurement was not approved by the analyst of the MP density measurement and one subject was withdrawn from the study before MP density measurement. Data from these 4 subjects were not included in the statistical analyses. Eight subjects were measured in the left eye instead of the right eye. Their measurements were included in the analysis, because there is a good correlation between both eyes (Hammond and Fuld, 1992). From the 187 adipose tissue obtained, the lutein concentrations of 21 samples were below the lower limit of quantification. Half of the limit of detection value was assigned to these samples and these data were included in the analyses. Data of 177 men and 199 women were used in the regression analyses after adjustment for age. Because of missing data in covariables, data of 175 men and 197 women were used in the multiple variable regression analyses, adjusted for age, total cholesterol, BMI and smoking status. In the two regression models for adipose lutein concentrations adjusted for age or age, BMI and smoking status 187 (89 men, 98 women) values were used.

Results

Study group characteristics

Study group characteristics and associations with serum lutein and MP density are presented in Tables 1 and 2. The 10^{th}, 50^{th} and 90^{th} percentiles of serum lutein concentrations were 0.10 μmol/L, 0.17 μmol/L and 0.27 μmol/L. The 10^{th}, 50^{th} and 90^{th} percentiles of serum zeaxanthin concentrations were 0.03 μmol/L, 0.05 μmol/L and 0.08 μmol/L. Iris color was not classified for all participants, but the group consisted mainly of people with blue or gray irises (category 1) (Table 1).

In men, we found significantly higher serum lutein concentrations in non-smokers in comparison with smokers.

Table 1 Baseline characteristics of volunteers and mean serum lutein concentrations and macular pigment (MP) density per category

Characteristics	*n*		Serum lutein[1]		MP density[1]	
	Men	Women	Men	Women	Men	Women
Total	177	199	0.16 ± 0.07	0.19 ± 0.08	0.35 ± 0.15[2]	0.31 ± 0.14
Smoking						
No	120	144	0.17 ± 0.07[3]	0.19 ± 0.09	0.35 ± 0.14	0.30 ± 0.14
Yes	57	55	0.15 ± 0.06	0.17 ± 0.08	0.34 ± 0.17	0.33 ± 0.14
Fruit and vegetable consumption						
Low	116	98	0.16 ± 0.06[4]	0.16 ± 0.06[4]	0.34 ± 0.14	0.30 ± 0.13
High	61	101	0.18 ± 0.07	0.21 ± 0.09	0.36 ± 0.17	0.32 ± 0.15
Iris color[5]						
Blue or gray	101	115	0.16 ± 0.07	0.19 ± 0.09	0.35 ± 0.14	0.31 ± 0.14
Blue, gray or green	28	24	0.15 ± 0.06	0.19 ± 0.06	0.33 ± 0.15	0.33 ± 0.15
Green or light brown	10	15	0.17 ± 0.07	0.17 ± 0.07	0.32 ± 0.17	0.28 ± 0.18
Brown	13	13	0.17 ± 0.07	0.19 ± 0.06	0.28 ± 0.14	0.33 ± 0.15
Dark brown	7	5	0.19 ± 0.06	0.22 ± 0.12	0.50 ± 0.19[6]	0.22 ± 0.06

[1] Mean ± SD. [2] Significantly different from women ($P < 0.05$). [3] Significantly different from smokers ($P < 0.05$). [4] Significantly different from high fruit and vegetable consumption ($P < 0.01$). [5] Data missing for 18 men and 27 women. [6] Significantly different from iris color category brown, Scheffé test.

Women had significantly higher total cholesterol and HDL concentrations than men (Table 2). Serum lutein concentrations correlated significantly with height (men), BMI (women), total cholesterol, HDL cholesterol and LDL cholesterol (both sexes) (Table 2).

Serum and blood concentrations were significantly lower in men than in women for α-tocopherol, vitamin C and all carotenoids but lycopene (Table 3). After adjustment for total cholesterol, serum β-carotene ($P = 0.07$) and α-tocopherol ($P = 0.42$) were not significantly different between men and women.

Table 2 Baseline characteristics in men ($n = 177$) and women ($n = 199$) and correlation with serum lutein (R lut) or macular pigment density (R mac)

Variable	mean ± SD		R lut[3]		R mac[4]	
	Men	Women	Men	Women	Men	Women
Age (y)	42 ± 15	41 ± 13	0.26[5]	0.30[5]	0.03	0.04
BMI (kg/m^2)[1]	24.5 ± 3.5	25.0 ± 4.9	−0.05	−0.24[5]	−0.21[5]	0.07
Height (m)	1.82 ± 0.08	1.69 ± 0.07[8]	−0.30[5]	−0.13[6]	−0.10	−0.06
Body weight (kg)[1]	81.4 ± 13.2	71.5 ± 14.9[8]	−0.21[5]	−0.28[5]	−0.23[5]	0.04
Total cholesterol (mmol/L)[2]	5.39 ± 0.96	5.67 ± 0.92[7]	0.20[5]	0.35[5]	−0.10	0.11
LDL cholesterol (mmol/L)[2]	3.82 ± 0.96	3.76 ± 0.92	0.15[5]	0.26[5]	−0.08	0.14[5]
HDL cholesterol (mmol/L)[2]	1.39 ± 0.31	1.76 ± 0.44[8]	0.21[5]	0.25[5]	0.07	−0.12
Triacylglycerols (mmol/L)[2]	1.20 ± 0.63	1.09 ± 0.49	−0.01	0.01	−0.18[5]	0.17[5]

[1] 176 men. [2] 198 women. [3] Correlation between serum lutein and characteristics. [4] Correlation between MP density and characteristics. [5] $P < 0.05$. [6] $P < 0.06$. [7] Significantly different from men; $P < 0.010$. [8] Significantly different from men; $P < 0.001$.

Lutein concentrations in adipose tissue were higher in women than in men ($P < 0.001$) (Table 3). The correlation between serum lutein and adipose lutein concentrations was 0.46 in men and 0.53 in women after adjustment for total cholesterol ($P < 0.001$). Serum lutein concentrations were significantly correlated to blood vitamin C, α-tocopherol and other serum carotenoids, except for lycopene in both men and women after adjustment for total cholesterol (Table 3).

Macular pigment density

Mean MP density in the total study group was 0.33 ± 0.15 (range 0.02 to 0.80). Mean MP density was 13% higher in men than in women ($P = 0.02$) (Table 1). In both men and women, MP density did not differ significantly between smokers and non-smokers or between the groups with high and low fruit and vegetable consumption. In men, MP density of iris category 5 was significantly higher than in iris category 4 (Table 1). MP density correlated significantly with BMI (men), body weight (men), LDL-cholesterol (women) and triacylglycerol (both sexes) (Table 2).

Table 3 Serum and adipose carotenoid, serum α-tocopherol and blood vitamin C concentrations in men ($n = 177$) and women ($n = 199$) and correlation with serum lutein (R lut) or macular pigment density (R mac)[1]

Variable	mean ± SD		R lut[3]		R mac[4]	
	Men	Women	Men	Women	Men	Women
Serum lutein	0.16 ± 0.07[6]	0.19 ± 0.08	–	–	0.37[5]	0.10
Serum zeaxanthin	0.05 ± 0.02[6]	0.05 ± 0.02	0.64[4]	0.64[4]	0.28[5]	0.07
Serum β-cryptoxanthin	0.17 ± 0.09[6]	0.22 ± 0.13	0.29[4]	0.46[4]	0.16[4]	0.02
Serum lycopene	0.35 ± 0.18	0.37 ± 0.18	0.001	0.14[5]	–0.01	0.07
Serum α-carotene	0.06 ± 0.06[6]	0.08 ± 0.06	0.38[4]	0.45[4]	0.25[4]	–0.004
Serum β-carotene	0.33 ± 0.19[6]	0.40 ± 0.25	0.30[4]	0.41[4]	0.23[4]	–0.05
Serum α-tocopherol	26.0 ± 5.5[6]	27.6 ± 6.6	0.23[4]	0.25[4]	0.04	0.21[4]
Blood vitamin C	40.2 ± 12.2[6]	49.9 ± 13.4	0.18[4]	0.29[4]	0.15[4]	0.20[4]
Adipose tissue lutein[7]	0.32 ± 0.17[6]	0.47 ± 0.31	0.46[4]	0.53[4]	0.29[4]	–0.007

[1] Lutein, β-cryptoxanthin, α-carotene, β-carotene, α-tocopherol in serum and fat biopsies were not normally distributed; variables were log-transformed before *t*-test and correlation analyses; μmol/L unless stated otherwise. [2] Partial correlation between serum lutein and other carotenoids and vitamins, adjusted for total cholesterol. [3] Partial correlation between MP density and carotenoids and α-tocopherol, adjusted for total cholesterol. Correlation between MP density and vitamin C and adipose tissue lutein is not cholesterol adjusted. [4] $P < 0.05$. [5] $P < 0.06$. [6] Significantly different from women. [7] Adipose tissue samples; μmol/kg wet weight: 89 men, 98 women.

In men, MP density correlated positively with serum lutein, zeaxanthin ($P < 0.06$), β-cryptoxanthin, α-carotene, β-carotene ($P < 0.05$), after adjustment for total cholesterol. Vitamin C was significantly correlated with MP density in men and women. The partial correlation between MP density and α-tocopherol adjusted for total cholesterol was significant in women. Serum lutein and zeaxanthin were not associated with MP density in women (Table 3). Furthermore, adipose lutein concentrations were positively associated with MP density in men but not women (Table 3).

Regression modeling

Serum lutein and zeaxanthin

Serum lutein and zeaxanthin appeared to be relevant predictors of MP density in men after adjustment for age (Models 1a and 1b, Table 4). In the model in which serum lutein and zeaxanthin were both included, serum lutein, but not zeaxanthin, predicted MP density in men (Model 1c, Table 4). In women, serum lutein and/or zeaxanthin were not associated with MP density (Models 1a-1c, Table 4).

BMI changed the magnitude of the regression coefficient of lutein both in men and women and was, therefore, included in the adjusted models. Smoking status changed the magnitude of the regression coefficient of serum lutein in women and was therefore included in the adjusted models of men and women. Total cholesterol was included in the multivariate model, because of the correlation between serum lipids and serum carotenoids. From the multivariate model with serum lutein and zeaxanthin (Model 2c, Table 4) we can conclude that a serum lutein increment of 0.1 μmol/L is associated with a 0.064 higher MP density (18% of the mean) in men after adjustment for age, total cholesterol, BMI and smoking status.

In women, serum lutein and zeaxanthin concentrations were not associated with MP density in the adjusted models (Model 2a-2c, Table 4). We calculated mean MP density values across quartiles of lutein divided by total cholesterol. In men, MP density was 0.28 ± 0.12, 0.31 ± 0.11, 0.37 ± 0.15 and 0.44 ± 0.17 across quartiles. In women, MP density was 0.31 ± 0.12, 0.30 ± 0.14, 0.30 ± 0.16 and 0.34 ± 0.15 across quartiles.

Table 4 Multivariate regression models to evaluate the association between serum lutein and/or zeaxanthin and macular pigment density

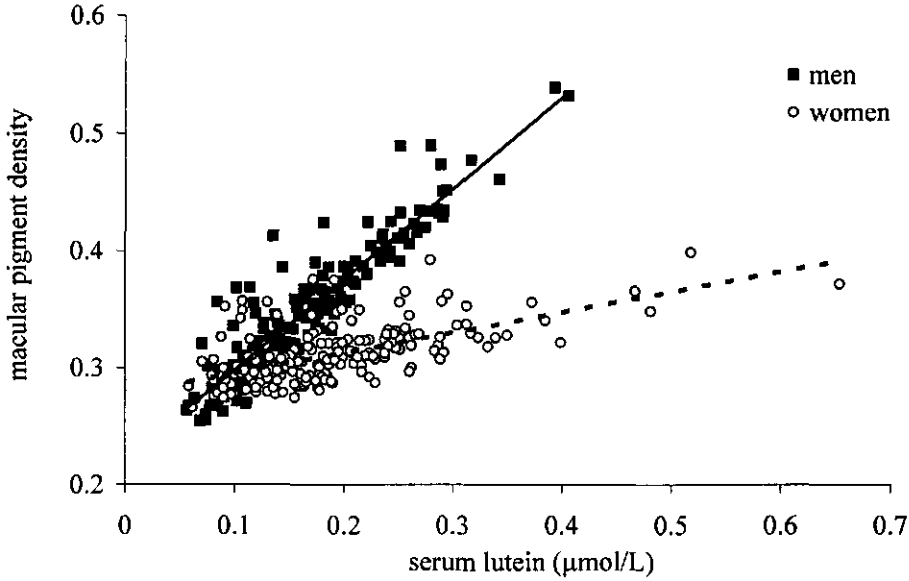
		Men		Women	
		β (SE)	R^2	β (SE)	R^2
Model 1a[1]	Lutein	0.081 (0.017)[5]	0.12	0.016 (0.013)	0.01
Model 1b[2]	Zeaxanthin	0.227 (0.062)[5]	0.07	0.062 (0.046)	0.01
Model 1c[3]	Lutein	0.071 (0.022)[6]	0.12	0.009 (0.017)	0.01
	Zeaxanthin	0.055 (0.082)		0.041 (0.060)	
Model 2a[4]	Lutein	0.080 (0.018)[5]	0.17	0.022 (0.014)	0.05
Model 2b[4]	Zeaxanthin	0.241 (0.065)[5]	0.14	0.068 (0.049)	0.04
Model 2c[4]	Lutein	0.064 (0.023)[6]	0.18	0.016 (0.017)	0.05
	Zeaxanthin	0.091 (0.083)		0.034 (0.061)	

[1] Model 1a: MP density = serum lutein (0.1 μmol/L) + age (years); 177 men, 199 women. [2] Model 1b: MP density = serum zeaxanthin (0.1 μmol/L) + age (years); 177 men, 199 women. [3] Model 1c: MP density = serum lutein (0.1 μmol/L) + serum zeaxanthin (0.1 μmol/L) + age (years); 177 men, 199 women. [4] Models 2a-c: adjusted for age (years), BMI (kg/m^2), total cholesterol (mmol/L), and smoking (0: no smoking; 1: < 1 cigarette/day; 2: 1-5 cigarettes/day; 3: 6-10 cigarettes/day; 4: 11-15 cigarettes/day; 5: > 15 cigarettes/day). [5] $P < 0.001$; [6] $P < 0.010$.

Figure 2 shows predicted individual MP density values with increasing serum lutein concentration for men and women separately after fitting a model with serum zeaxanthin, age, total cholesterol, BMI and smoking status. These regression lines are based on a model based on the total group, including sex and the sex × lutein interaction term and confounders as independent variables.

After adjustment for other serum carotenoids, vitamin C and α-tocopherol, the regression coefficient of serum lutein was lower (0.053 ± 0.025) in men, but did not change in women (0.016 ± 0.018). In women, vitamin C was positively associated with MP density (0.02 ± 0.008 per 10 μmol/L increment).

Figure 2 Predicted individual MP density values for men (squares) and women (circles) after fitting a model with zeaxanthin, age, smoking status, total cholesterol and BMI with increasing serum lutein concentrations. The dotted line represents the predicted MP density for women and the solid line represents men[1]



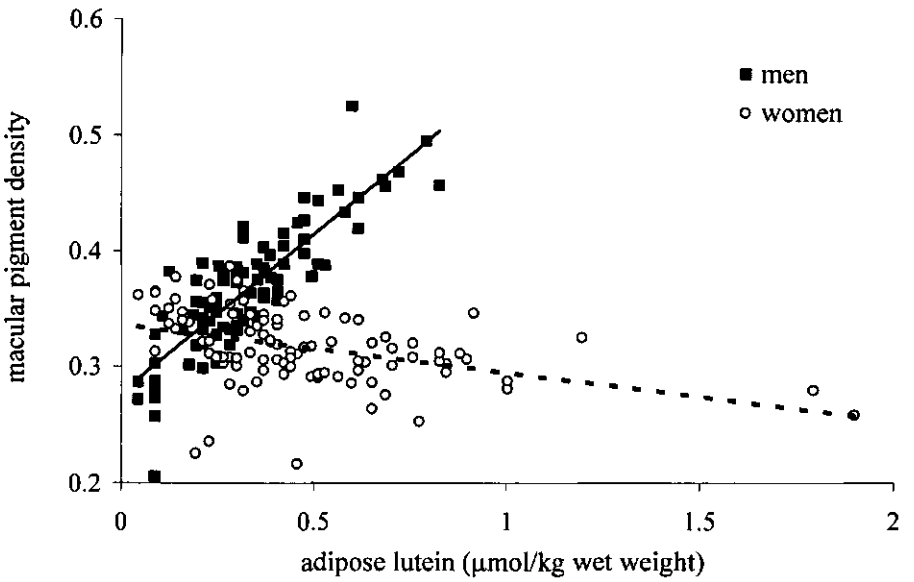
[1] Multivariate model (total population): MP density = serum lutein + serum zeaxanthin + age (years) + total cholesterol (mmol/L) + BMI (kg/m^2) + smoking (categories) + sex + sex × serum lutein. Interaction term: $P < 0.01$.

A possible explanation for the absence of a significant correlation among women is the heterogeneity of the female group, which included both pre- and postmenopausal women. Plasma carotenoids fluctuate by phase of the menstrual cycle (Forman *et al.*, 1996). To evaluate whether these fluctuations did influence individual carotenoid concentrations, we divided the group into women < 50 years (n = 143) or ≥ 50 years (n = 56) and included an interaction term (older or younger then 50 years × lutein) in the model. The regression coefficients of lutein and zeaxanthin were not associated with MP density in both pre- and postmenopausal women.

Adipose lutein

Next, the association between adipose lutein concentrations and MP density was investigated. In accordance with serum lutein, adipose lutein concentrations were positively associated with MP density in men, but not women. The regression coefficients for adipose lutein after adjustment for age were 0.028 ± 0.010 (P = 0.01) and –0.006 ± 0.005 (P = 0.25) per increment of 0.1 μmol/kg wet weight in men and women, respectively. BMI and smoking status changed the magnitude of the regression coefficients in men and were both included. After adjustment for age, BMI and smoking status the regression coefficients of adipose lutein were in men 0.021 ± 0.010 (P = 0.04) and in women –0.006 ± 0.005 (P = 0.27). Figure 3 shows the predicted individual MP density values with increasing adipose lutein concentrations after fitting a model with age, BMI and smoking status, for men and women, separately.

Figure 3 Predicted individual MP density values for men (squares) and women (circles) after fitting a model with age, smoking status and BMI with increasing adipose tissue lutein concentrations. The dotted line represents the predicted MP density for women and the solid line represents men[1]



[1]Multivariate model: MP density = adipose lutein + age (years) + smoking (categories) + BMI (kg/m^2) + sex + sex × adipose lutein. Interaction term: $P < 0.01$.

To compare the subjects from whom we took an adipose sample and the total group, we evaluated the association between serum lutein and MP density in the subgroup in whom we had collected adipose tissue samples (89 men, 98 women). After adjustment for age, total cholesterol, BMI and smoking, the coefficients of serum lutein were higher in men ($\beta = 0.088$, SE = 0.026, $P < 0.01$, $n = 89$) and in women ($\beta = 0.049$, SE = 0.024, $P = 0.04$, $n = 97$) in comparison with the total male and female group. In this subgroup, the associations between serum lutein and MP density in women are weaker than in men. The interaction term serum lutein × sex was not significant, which suggests that the associations were not significantly different between men and women. We evaluated the difference in confidence intervals of the regression coefficients of serum lutein between the subgroup of women of whom we had adipose tissue samples and the remaining female subjects (n =101). The regression coefficients of serum lutein in these two groups were 0.049 (95% CI 0.0020 – 0.096) and 0.0025 (–0.033 – 0.038), respectively. Exclusion of samples below the detection limit did not change the results.

Discussion

This study was conducted to assess the cross-sectional associations between the serum carotenoids lutein and zeaxanthin and MP density. In addition to biomarkers reflecting short-term (serum) intake of carotenoids, we also evaluated, as a biomarker of long-term intake, the associations of lutein in adipose tissue with MP density.

Serum and adipose concentrations of lutein are significantly higher in women, but their MP density is lower than in men. The relationships of serum lutein, serum zeaxanthin and adipose lutein with MP density are higher in men than in women.

The strength of this study was the large study group. This large study group enabled us to compare men and women. MP density was been assessed with an objective non-invasive measuring technique, in contrast to most other studies, which used a technique that was rather demanding for subjects (Hammond *et al.*, 1996a; Johnson *et al.*, 2000; Landrum *et al.*, 1997b; Hammond *et al.*, 1997; Hammond *et al.*, 1996b).

There were several limitations of this study that may attenuate the results of this study. We aimed to select Dutch volunteers with high and low fruit and vegetable consumption in order to reach a wide range in carotenoid exposure. In the end, the

range of serum lutein concentrations in men and women was 0.06 – 0.41 μmol/L and 0.06 – 0.65 μmol/L, respectively. These ranges are approximately similar to the ranges of serum lutein concentrations of male and female subjects in the Netherlands with ranges of 0.07 – 0.42 μmol/L and 0.08 – 0.51 μmol/L, respectively (Olmedilla *et al.*, 2001). However, serum lutein concentrations in subjects from the United States or South European countries (Spain and Italy) are higher than in the Netherlands (Mares-Perlman *et al.*, 2001; Hammond *et al.*, 1996a; Olmedilla *et al.*, 2001). In a global context, we have to consider that the range in serum carotenoids in the present study is narrow in comparison to the differences in carotenoid concentrations between populations by the interpretation of our results. This difference is possibly caused by the intake of supplements, fortified foods or fruit and vegetables.

We did not perform duplicate MP density measurements in each subject. A previous study showed a within subject variation coefficient of 17% (Berendschot *et al.*, 2000). This measurement error could have attenuated the associations between concentrations of carotenoids in serum, adipose tissue and macula pigment density. A possible source of bias is that we allocated volunteers to the adipose tissue sampling group. They had the possibility to refuse this measurement. When comparing the volunteers with or without data on adipose tissue lutein concentrations, we observed that men who gave an adipose tissue sample had somewhat higher MP density values. But no differences in other characteristics, including BMI, were observed between the group with and without data on adipose tissue lutein concentrations in either men or women. We evaluated the association between serum lutein and MP density in the subgroup in which we had collected adipose tissue samples and compared it with the associations in the total study group. In women, the differences in these associations between the total group and subgroup is difficult to explain and possibly due to chance or caused by selection bias.

In contrast with other studies (Hammond *et al.*, 1996b; Hammond and Caruso-Avery, 2000), we did not find associations between smoking status and MP density. A possible explanation for this discrepancy is the classification of smoking status in categories of cigarettes per day. We did not have information on smoking duration or smoking history. The covariate 'iris color' was not associated with MP density in the total population, contrary to results of two studies that found

significant differences in MP densities among iris categories (Hammond *et al.*, 1996c; Hammond and Caruso-Avery, 2000). Our volunteers fell predominantly in iris color category 1 (blue/gray). However, we found a positive association between iris color and MP density in men. This was largely attributable to men in iris category 5 (dark brown) with a high MP density (0.50 ± 0.19). We did not find an association with age. Another study found a slight decline of MP density with age (Hammond and Caruso-Avery, 2000).

A previous study (Hammond *et al.*, 1996a) also found stronger correlations between plasma lutein and MP density for men (r = 0.62; P < 0.005) than for women (r = 0.3, P < 0.05). The baseline results of a study with lutein supplementation showed that plasma lutein concentrations were highly correlated with MP density (r = 0.82, P < 0.001) in men (Berendschot *et al.*, 2000). Our results are in line with the sex differences found in other studies, but our correlations are generally weaker. Past studies with feeding extra lutein sources have shown an increase in macular pigment density in women (Hammond *et al.*, 1997). The narrow range of serum lutein concentrations in the present study, relative to past studies may explain the weaker associations between serum lutein and MP density in this study. In cross-sectional studies variables are determined at one point in time. Therefore, it is difficult to assess the temporal relationship between nutritional factors, for example serum lutein and the dependent variable, in this case MP density. Furthermore, our study group consisted of both pre- and postmenopausal women and serum carotenoids fluctuate by phase of menstrual cycle (Forman *et al.*, 1996; Forman *et al.*, 1998). Therefore, the serum lutein concentrations in women may be a less good reflection of the past lutein intake. However, serum lutein was not associated with MP density in postmenopausal women. By comparison of MP density values across quartiles of lutein divided by total cholesterol, MP density showed a linear relation in men, but not in women. In women, MP density was higher in the last quartile of serum lutein adjusted for total cholesterol. This could suggest that in women serum lutein is associated with MP density only at high serum lutein concentrations.

Blood vitamin C concentrations were positively associated with MP density in women. Vitamin C is abundant in the eye, including the retina and the lens. Vitamin C may participate with other antioxidants in electron transfer reactions to reduce oxidative stress in the eye, but the exact mechanism is not evident (Mares-

Perlman and Klein, 1999). We hypothesize that because of this antioxidant capacity, vitamin C is positively associated with MP density. It could spare the antioxidants lutein and zeaxanthin in the MP. Furthermore, this positive association between vitamin C and MP density could reflect that vitamin C and carotenoids travel in the same foods. Therefore, vitamin C could function as marker of carotenoid intake. Observational epidemiological studies are inconclusive as to the role of vitamin C in the prevention of AMD (Seddon *et al.*, 1994; Mares-Perlman and Klein, 1999).

Both, concentrations of fat-soluble vitamins in adipose tissue and plasma (serum) concentrations could be used as markers of dietary intake. Since both take into account variations in absorption and metabolism, it may be better to talk about markers of internal dose instead of dietary intake (Kardinaal *et al.*, 1995). The results of the association between adipose tissue concentrations and MP density were in line with the associations between serum lutein concentrations and MP density in both men and women. Lutein and zeaxanthin are dynamic components of tissues and therefore the metabolism of lutein may differ between men and women (Johnson *et al.*, 2000). A previous study showed a negative correlation between MP density and adipose tissue lutein in women, but a positive one in men (Johnson *et al.*, 2000). However, this study was limited by the small number of subjects studied (13 women, 8 men). In our study with a larger number of adipose tissue samples (89 men, 98 women), we found a positive association between adipose tissue lutein and MP density in men and a slightly negative association in women (Figure 3). Because of this slightly negative association between adipose lutein concentrations and MP density in women, our findings do not contradict the suggestion that adipose tissue competes with macular pigment for carotenoids more effectively in women than in men (Johnson *et al.*, 2000). As far as we know, the exact mechanism of uptake of lutein and zeaxanthin in the retina has remained elusive.

In summary, men had lower concentrations of serum and adipose lutein, but had a higher MP density. From these findings we can conclude that a serum lutein increment of 0.1 μmol/L is associated with a 0.064 higher MP density (18% of the mean) in men. We suggest, therefore, that dietary intervention to increase MP density may have effect in men. In women, no significant association between serum lutein and MP density was found in the total female group. In the female

group that gave an adipose tissue sample, a positive association between serum lutein and MP density was found, but this association was weaker than in men. Adipose tissue could be an important factor in the association between serum lutein and MP density. Carotenoid concentrations in adipose tissue have to be taken into account to elucidate the influence of lutein intake on MP density. If MP density has a protective effect against AMD, our results in the total population are in line with the notion that men are at lower risk for AMD than women (Beatty *et al.*, 1999). Dietary intervention studies are necessary to investigate the influence of lutein intake on MP density and AMD in men and women.

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Chapter 3

Lutein and zeaxanthin in the eye may retard aging of the lens

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Submitted

Abstract

Objective: To investigate, whether nutritional factors and possible risk factors for cataract influence the density of the lens (LD).

Design: 376 subjects, aged 18–75 years were recruited. In a cross-sectional design, serum was analyzed for lutein, zeaxanthin, vitamin C, α-tocopherol and cholesterol. Adipose tissue (n = 187) was analyzed for lutein. Lens density (LD) and macular pigment (MP) density were measured by spectral fundus reflectance.

Results: LD at 420 nm was 0.52 ± 0.17. It showed a significant association with age (β = 0.0085, $P < 0.001$) and MP density (β = –0.096, $P = 0.019$). For subjects below 50 years (LD = 0.45 ± 0.11), we found only a single significant β-coefficient for age of 0.0063 ($P < 0.001$). For subjects above 50 years (LD = 0.68 ± 0.16), we found significant β-coefficients for age of 0.011 ($P < 0.001$) and for MP density of –0.25 ($P = 0.005$). Controlling for age, we found no associations between LD and other possible risk factors of age-related cataract, nor with serum or adipose tissue concentrations of carotenoids, vitamin C and α-tocopherol.

Conclusions: Macular pigment is composed of lutein and zeaxanthin, the only carotenoids that have been found in human lenses. Inverse relationship between LD and MP density suggests that lutein and zeaxanthin may retard aging of the lens.

Introduction

Age-related cataract is the leading cause of visual disability (Apple *et al.*, 2000). A number of nutritional determinants possibly influence age-related cataract. Vitamins C and E and the carotenoids lutein and zeaxanthin have been shown to be present in human lenses, with vitamin C at levels several fold that of plasma (Yeum *et al.*, 1995; Bates *et al.*, 1996; Yeum *et al.*, 1999). They all can reduce oxidative stress, which has been shown to influence the development of cataract (Gerster, 1989; Bunce *et al.*, 1990; Jacques and Taylor, 1991). Low vitamin C content in the lens appeared to be a good indicator of cataract severity (Tessier *et al.*, 1998).

Plasma levels of vitamin C were not associated with risk of cataract (Libondi *et al.*, 1991; Vitale *et al.*, 1993; Gale *et al.*, 2001). Studies on a possible association between plasma levels of vitamin E and risk of cataract showed conflicting results (Libondi *et al.*, 1991; Vitale *et al.*, 1993; Gale *et al.*, 2001; Lyle *et al.*, 1999a). Self-reported consumption of vitamins C and E supplements was shown to be associated with reduced risk of cataract (Robertson *et al.*, 1991; Jacques and Chylack, 1991; Mares-Perlman *et al.*, 1995a; Simon and Hudes, 1999). However, studies on consumption of vitamin C supplements showed controversial results (Hankinson *et al.*, 1992a; Jacques *et al.*, 1997; Van der Pols, 1999; Mares-Perlman *et al.*, 2000; Chasan-Taber *et al.*, 1999a). Conflicting results were also reported for multivitamin or vitamin E use (Leske *et al.*, 1991; Leske *et al.*, 1998; AREDS, 2001).

Intake of foods, rich in lutein and zeaxanthin like spinach and broccoli, was associated with a lower relative risk of cataract (Lyle *et al.*, 1999a; Hankinson *et al.*, 1992a; Tavani *et al.*, 1996; Brown *et al.*, 1999; Chasan-Taber *et al.*, 1999b; Lyle *et al.*, 1999b; Mares-Perlman *et al.*, 1995b). Lutein and zeaxanthin are also components of macular pigment (MP) in the eye (Bone *et al.*, 1988; Handelman *et al.*, 1988). A significant association has been found between MP density and concentrations of serum and adipose tissue lutein (Berendschot *et al.*, 2000; Bone *et al.*, 2000), be it that there are significant differences between men and women (Hammond *et al.*, 1996; Johnson *et al.*, 2000). Further, intake of lutein modifies the MP density (Berendschot *et al.*, 2000; Hammond *et al.*, 1997a; Landrum *et al.*, 1997). MP density is probably a better measure of the lutein and zeaxanthin content in the eye, than serum and adipose tissue values of carotenoids.

Besides nutritional determinants, other factors might influence cataract. Smoking has been identified to increase cataract risk (Simon and Hudes, 1999; Leske *et al.*, 1991; West *et al.*, 1989; West *et al.*, 1992; Hankinson *et al.*, 1992b; Klein *et al.*, 1993; West *et al.*, 1995; Christen *et al.*, 2000). However, smoking is also associated with diminished carotenoid and vitamin C status (Russell-Briefel *et al.*, 1985; Schectman *et al.*, 1989), and there appeared to be a diminished risk for cataract in smokers who used multivitamins in comparison with smokers not using multivitamins (Seddon *et al.*, 1994). Both height and abdominal adiposity have been shown to be independent risk factors for cataract (Caulfield *et al.*, 1999; Schaumberg *et al.*, 2000). The association between body mass index (BMI) and cataract is controversial (Leske *et al.*, 1991; Mohan *et al.*, 1989; Tavani *et al.*, 1995; Hankinson *et al.*, 1993; Glynn *et al.*, 1995; Hiller *et al.*, 1998; Klein *et al.*, 1998a). Subjects with darker colored irises tend to have higher risk of cataract (Collman *et al.*, 1988; Taylor *et al.*, 1988; The Italian-American Cataract Study Group, 1991). Finally, women have a somewhat higher incidence of cataract than men (Leske *et al.*, 1997; Klein *et al.*, 1998b; Chen *et al.*, 1986; Klein and Klein, 1982).

There is a strong relation between lens aging and cataract, the latter being the end stage of deterioration of the aging lens (Sample *et al.*, 1988; Young, 1991; Bron *et al.*, 2000). Lens senescence can be quantified by the increase in lens density, which has been studied extensively (Sample *et al.*, 1988; Pokorny *et al.*, 1987; Johnson *et al.*, 1993; Delori and Burns, 1996; Van Norren and Vos, 1974; Cook *et al.*, 1994; Gaillard *et al.*, 2000). Although these studies showed strong positive associations between lens density and age, there are substantial individual differences at each age. The aim of this study was to investigate, whether nutrition and possible risk factors for cataract mentioned above, could account for part of these individual differences. Fundus reflectance has been shown to yield reliable estimates of the lens density (Delori and Burns, 1996; Van de Kraats *et al.*, 1996). It was used in this study, having the advantage that the density of the macular pigment was obtained simultaneously.

Methods

Subjects

The subjects in our study were recruited from the pool of volunteers of TNO Nutrition and Food Research and through advertizing in local and regional newspapers and on television. The cross-sectional data analyzed here were gathered during the baseline measurements of an intervention study designed to test an unrelated hypothesis. Advertizing aimed at recruiting people who have a general interest in participating in nutrition studies. Respondents who expressed potential interest received a questionnaire eliciting data on daily fruit and vegetable consumption and lifestyle factors. This questionnaire was designed for ranking subjects according to their intake and not for quantitative measurements. Respondents in the highest quintile of fruit and vegetable consumption and those in the lowest tertile (calculated in the total group) were invited for an oral briefing and screening. In the lowest stratum vitamin supplement users were excluded. The major inclusion criteria were age between 18–75 years, and consumption of an average Western diet. The main exclusion criteria were pregnancy and/or lactation, or wishing to become pregnant, serum cholesterol > 7.5 and/or triacylglycerol > 2.3 mmol/L if not under stabilized hypercholesterolemia/hyperlipidemia treatment, and anticoagulant therapy. A total of 380 volunteers entered the study. Two volunteers squinted too much during the reflectance measurement. Further, one measurement failed for other reasons and one subject withdrew from the study before the reflectance measurement. Data from these 4 subjects were not included in the statistical analyses. Eight subjects were measured in the left eye instead of the right eye. Their measurements were included in the analysis, because there is a good correlation between both eyes (Hammond and Fuld, 1992). The final study group comprised 177 men and 199 women. The study was performed according to ICH (International Conference on Harmonization of Technical Requirements of Registration of Pharmaceuticals for Human Use) guidelines for good clinical practice, and was approved by an external Medical Ethical Committee. Informed consent was obtained from all subjects.

Blood and adipose tissue sampling

Blood samples from subjects were obtained between 800 and 930h after an overnight fast. For the analysis of carotenoids and α-tocopherol blood was

collected in tubes containing clot activator and gel (Becton Dickinson, Vacutainer systems). These tubes were immediately stored in a closed box, to avoid breakdown of carotenoids by UV light. Tubes were centrifuged within 15–30 min after collection at approximately 2000 × *g* for 10 min at ca. 4°C to obtain serum. After centrifugation serum was removed and stored at ca. –80°C. Before storage, all serum was handled in subdued light.

For the analysis of vitamin C, blood was collected in tubes containing lithium heparin (Becton Dickinson, Vacutainer systems). A 0.5 mL aliquot of blood was added to 2 mL metaphosphoric acid (50 g/L; Mallinckrodt Baker, Deventer, The Netherlands) before freezing to preserve the vitamin C concentration during storage. This mixture was stored at ca. –80°C.

For the analysis of total cholesterol, blood was collected in tubes containing clot activator and gel (Becton Dickinson, Vacutainer systems). Tubes were centrifuged within 15–30 min after collection at ca. 2000 × *g* for 10 min at ca. 4°C to obtain serum. After centrifugation serum was removed and stored at ca. –20°C.

Subcutaneous adipose tissue was obtained by needle biopsy from the lateral buttock (Beynen and Katan, 1985), using a 16-gauge needle attached to a plastic container in which the tissue was collected by connecting a vacuum tube. Samples were kept in the plastic container, immediately placed on dry ice and stored at ca. –80°C.

Chemical analyses

All carotenoids, α-tocopherol and vitamin C were analyzed by HPLC. The coefficient of variation (CV) for the analysis of lutein, zeaxanthin and α-tocopherol were 3.7%, 11.5%, 2.8%, respectively (at mean concentrations of 0.16, 0.03, 20.9 μmol/L in the quality control samples, respectively). The CV for the analysis of vitamin C was 10%. Total cholesterol was analyzed by enzymatic conversion to a stable chromogen, which could easily be detected by colorimetry (commercial available kit; Boehringer, Mannheim, Germany). For more details see Broekmans *et al.* (in press).

Measurement of density of the lens and the macular pigment

Spectral fundus reflectance was measured with the Utrecht Retinal Densitometer (Van Norren and Van de Kraats, 1989). Briefly, a rotating wheel (14 revolutions

per second) offered a sequence of 14 interference filters in the range 430 to 740 nm to enable quasi-simultaneous measurement of the reflectance across the visual spectrum. Light reflected from the fundus was measured in a detection field of 1.5°, concentric within the illumination field of 1.8°. To obtain an estimate of the mean lens and MP density in this area, spectral fundus reflectance was measured in two conditions: (1) with the instrument's entry and exit pupils aligned to the peak of the Stiles-Crawford (SC) function, and (2) 2 mm temporal to the SC peak (Van de Kraats *et al.*, 1996). A detailed optical model of foveal reflection was used to arrive at individual estimates of parameters as equivalent thickness of blood layer and densities of MP, lens and melanin (Van de Kraats *et al.*, 1996). In short, the incoming light was assumed to reflect at the inner limiting membrane, the disks in the outer segments of photoreceptors and the sclera. Using known spectral characteristics of the different absorbers within the eye (lens, MP, melanin, blood), the densities of the pigments and percent reflectance at the interfaces were optimized, to fit the measured data at all wavelengths. The spectral characteristic of the lens density was composed of a non-aging and an aging part (Pokorny *et al.*, 1987). Only the aging part was optimized in the fit procedure. The subjects' pupils were dilated with tropicamide 0.5%.

Measurement of other characteristics

Smoking status was obtained by a questionnaire. Iris color was graded by a set of four standard photographs, providing a five-grade classification system (Seddon *et al.*, 1990). Weighing the subject wearing indoor clothing, without shoes, wallet and keys, assessed body weight. Height was measured without shoes. Body mass index (BMI) was calculated with the formula body weight (kg)/ body height squared (m^2).

Statistical analyses

The SPSS statistical software package (Version 8.0.2) was used for data analysis. Pearson correlation coefficients were calculated between continuous variables. To adjust for age effects we also calculated partial correlation coefficients, controlling for age. Students' T-tests and ANOVA were used to evaluate possible differences in continuous variables between men and women, between smokers and non-smokers, between subjects with high and low fruit and vegetable intake, and

between subjects with the different iris colors. Chi-square tests were used to evaluate possible differences between categorical baseline characteristics (gender, iris color, smoking, fruit and vegetable intake). If variables were not normally distributed, they were log-transformed. Regression analysis was used to evaluate the association between categorical variables (gender, iris color, smoking), covariates (age, MP density, vitamins, lutein, zeaxanthin, cholesterol) and lens density.

Results

Mean lens density was 0.52 ± 0.17. Study group characteristics are presented in Tables 1 and 2. For ranking, pre-study participants were classified into those with a diet low in fruit and vegetables, fruit juices included (57% of the study sample) and those with diet rich in these foods (43% of the study sample). The latter group consisted of significantly more women (62%) than the former (46%, $P = 0.001$). The percentage of male smokers (32%) did not differ from that of female smokers (28%; $P = 0.33$). Lens density did neither differ significantly between men and women, nor between the groups with high and low fruit and vegetable intake, nor between smokers and non-smokers (Table 1).

Iris color was classified in 331 participants. The participants predominantly (65%) had blue or gray irises. The distribution of subjects in the five iris categories did not differ between men and women ($P = 0.72$). Lens density did not differ among the iris categories (Table 1).

Serum and blood concentrations were significantly lower in men than in women for lutein and zeaxanthin, vitamin C, and total cholesterol. Lutein concentration in adipose tissue was also significantly lower in men than in women. MP density was significantly higher for men than for women (Table 2)

Age correlated most significantly with lens density ($r = 0.71$, $P < 0.001$), explaining 50% of the variance. Further, serum and adipose lutein, serum α-tocopherol, height, BMI and total cholesterol also correlated significantly with lens density (Table 2, column 5). Other previously mentioned characteristics (MP density, serum zeaxanthin and blood vitamin C) showed no association with lens density. We also found a significant correlation between age and serum lutein, serum α-tocopherol, adipose tissue lutein, BMI, height and total cholesterol (Table 2, column 6). Therefore, we calculated the partial correlation coefficient between

all parameters and lens density, controlling for age (Table 2, column 7). All aforementioned correlations disappeared.

Table 1 Characteristics and mean lens optical density at 420 nm (± SD) per category

	n	Lens optical density	*P*-value
Gender			
Men	177	0.52 ± 0.17	0.98
Women	199	0.52 ± 0.16	
Smoking			
No	264	0.52 ± 0.17	0.66
Yes	112	0.52 ± 0.17	
Fruit and vegetable consumption[1]			
Low	214	0.51 ± 0.15	0.16
High	162	0.54 ± 0.19	
Iris color			
Blue or gray	216	0.52 ± 0.17	0.42
Blue, gray or green	52	0.53 ± 0.18	
Green or light brown	25	0.57 ± 0.19	
Brown	26	0.55 ± 0.14	
Dark brown	12	0.46 ± 0.16	

[1] obtained pre-study.

Only MP density showed a small significant negative correlation with lens density ($r = -0.12$, $P = 0.019$). This could imply that MP density provided more detailed information on lutein/zeaxanthin content in the lens than serum lutein, serum zeaxanthin and adipose tissue lutein. To test this hypothesis, we used linear regression analysis to identify factors that are associated most significantly with lens density. We used the forward conditional method with default selection criteria, $P < 0.05$ to enter and $P > 0.10$ to remove. Age, serum lutein, serum zeaxanthin, adipose tissue lutein, MP density, serum vitamin C and α-tocopherol were used as independent variables and lens density as dependent variable. Total cholesterol was included, because of its correlation with serum carotenoids, α-tocopherol, height and BMI. Height, BMI, gender, smoking and iris color were also included.

We only found significant β-coefficients for age of 0.0085 ± 0.0004 ($P < 0.001$; CI: 0.0076, 0.0094; 16% per 10 years) and for MP density of −0.096 ± 0.041 ($P = 0.019$; CI: −0.177, −0.016; −2.7% per SD in MP density).

Table 2 Descriptive characteristics (mean ± SD) for the total study group and men and women separately

	Total ($n = 376$)	Men ($n = 177$)	Women ($n = 199$)[a]	r[b]	r[c]	r[d]
Age (y)	42 ± 14	42 ± 15	41 ± 13	0.71[3]		
MP density (at 460 nm)	0.33 ± 0.15	0.35 ± 0.15	0.31 ± 0.14[1]	−0.06	0.037	−0.12[1]
Serum lutein (μmol/L)	0.18 ± 0.08	0.16 ± 0.07	0.19 ± 0.08[2]	0.19[3]	0.26[3]	0.003
Serum zeaxanthin (μmol/L)	0.049 ± 0.020	0.046 ± 0.018	0.052 ± 0.023[2]	0.06	0.051	0.039
Blood vitamin C (μmol/L)	45 ± 14	40 ± 12	50 ± 13[3]	0.03	−0.048	0.090
Serum α-tocopherol (μmol/L)	26.8 ± 6.1	26.0 ± 5.5	27.6 ± 6.6[1]	0.30[3]	0.45[3]	−0.030
Adipose tissue lutein (μmol/kg wet weight)[e]	0.40 ± 0.26	0.32 ± 0.17	0.47 ± 0.31[3]	0.12[1]	0.05	0.12
Height (m)	1.75 ± 0.10	1.82 ± 0.08	1.69 ± 0.07[3]	−0.18[2]	−0.21[3]	−0.047
BMI (kg/m^2)	24.7 ± 4.3	24.5 ± 3.5	25.0 ± 4.9	0.14[2]	0.22[3]	−0.021
Total cholesterol (mmol/L)	5.53 ± 0.95	5.39 ± 0.96	5.67 ± 0.92[2]	0.34[3]	0.45[3]	0.043

[1]Significant at the $P = 0.05$ level, [2]Significant at the $P = 0.01$ level, [3]Significant at the $P = 0.001$ level. [a]Significance values refer to a comparison between men and women with the Students' T-test. [b]Correlation with lens optical density. [c]Correlation with age. [d]Correlation with lens optical density, controlling for age (partial correlation). [e]Adipose tissue lutein was only determined in 187 subjects (men: $n = 89$, women: $n = 98$).

Hammond *et al.* (1997b) observed for older subjects ($r = -0.47$, $n = 39$, range 48–82 years) also an inverse relationship between lens density and MP density. For younger subjects ($r = 0.07$, $n = 12$, 24–36 years) they found no relationship. We added an interaction term age × MP density in the regression analysis, but it did not contribute significantly. We also stratified our data (under and above 50 years) and applied the regression analysis on both strata. Lens density was 0.68 ± 0.16 for the

older subjects and 0.45 ± 0.11 for the younger subjects. MP density was 0.34 ± 0.16 and 0.32 ± 0.14 respectively. For the older subjects (n = 114) we found significant β-coefficients for age of 0.0111 ± 0.0021 (P < 0.001; CI: 0.007, 0.015) and for MP density of –0.240 ± 0.086 (P = 0.005; CI: –0.410, –0.071; –5.6%). For the younger subjects (n = 262) we only found a significant β-coefficient for age of 0.0063 ± 0.0007 (P < 0.001; CI: 0.0041, 0.0077), and no association between lens density and MP density.

Discussion

As expected, lens density increased significantly with age. MP density showed an inverse relationship with lens density after adjustment for age. Like others, we found a significantly higher MP density in men than in women, despite lower serum and adipose tissue levels (Hammond *et al.*, 1996; Hammond *et al.*, 2000a). Further, like others, we also found a higher correlation between MP density and concentrations of serum and adipose tissue lutein in men than in women (Hammond *et al.*, 1996; Johnson *et al.*, 2000). Thus, the ability to transport lutein and zeaxanthin from the blood into the eye seems less in women than in men. It may be the reason that we found lens density to show an inverse association with MP density, rather than with serum and adipose tissue values of lutein and zeaxanthin. It may also be an explanation for the higher incidence of cataract in women than in men, apart from the impact of oestrogen (Snow and Seddon, 2000).
Similar to Hammond *et al.* (1997b) we stratified our data (under and above 50 years). Only for the older subjects we found a significant association between lens density and MP density. To study this in more detail, we added an interaction term age × MP density in the regression analysis. However, the interaction term did not contribute significantly. It could be due to a nonlinear dependence of the lens density on age, not accounted for in the linear model analysis. Pokorny *et al.* (1987) determined, for subjects after age 60, a threefold increase in the rate at which the lens density changes as a function of age. In this study we found the β-coefficient for age to be 1.76 ± 0.39 times larger for subjects older than 50 years, than for the younger subjects. For a cut point at age 60 we found the β-coefficient for age to be 2.09 ± 0.81 times larger for older subjects than for the younger subjects. We added an extra quadratic age term in General Linear Model analysis, which indeed turned out to contribute significantly. However, the age × MP density

interaction term remained non-significant. Thus, in the total study group we could not find a significant change in the regression coefficient for the MP density with age, although in the stratified data the regression coefficient was only significant for the older subjects.

The inverse association between MP density and lens density could reflect the greater likelihood of subjects with high MP density to have other healthy behaviours that retard lens aging. We found a significant correlation between MP density and serum vitamin C ($r = 0.12$, $P = 0.021$). However, serum vitamin C and other markers for a healthy lifestyle, and which are possible risk factors for age-related cataract, like BMI and smoking, have been included in the regression analysis. Only MP density showed a significant contribution.

We found no difference in lens density between smokers and non-smokers. Hammond *et al.* (1999) studied lens density in relation to smoking behavior in younger subjects ($n = 41$), without cataract. They found a significant dose-response relationship between smoking frequency and lens density. Their data indicate that smoking is directly related to age-related increases in lens density throughout life, and that these increases persist even after smoking cessation. We had no information on duration of smoking and also no information of ex-smokers. This may have lessened the strength of our analysis.

Hammond *et al.* (2000b) also found significantly higher lens optical densities in subjects with dark irises in comparison with subjects with light irises. We similarly divided our study group and found a mean lens density of 0.52 ± 0.17 for subjects with light irises ($n = 268$, the first two categories) and of 0.54 ± 0.17 for subjects with dark irises ($n = 63$, the last three categories). Although lens density was higher in subjects with dark irises, this difference was not significant ($P = 0.42$). Hammond *et al.* (2000b) found only differences in lens density between light and dark irises for older subjects (> 45 years). Younger subjects showed no differences in lens density. We could not find any age effect in our study group. Model analysis of the reflectance spectra yielded a retinal melanin density of 0.87 ± 0.18 for subjects with light irises and of 1.02 ± 0.27 for subjects with dark irises ($P < 0.001$). The spectral absorbance of melanin and the aging component of the lens density both show a slow decrease with increasing wavelength, without any characteristic spectral features. However, due to choroidal blood absorption, melanin shows its spectral fingerprint only for wavelengths > 600 nm, whereas the

lens density is determined for wavelengths < 600 nm. As a consequence their absolute values can be determined independently and reliably, and there is no indirect covariance between iris color and lens density form our parameter fitting.
In summary, lens density showed a significant association with age and MP density. MP is composed of lutein and zeaxanthin, the only carotenoids that have been found in human lenses. MP density is associated with serum lutein and adipose lutein and can be modified by lutein intake. Inverse relationships between MP density and lens density suggest that lutein and zeaxanthin in the eye may retard aging of the lens.

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Chapter 4

Determinants of skin sensitivity to solar irradiation

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Submitted

Abstract

Background: Acute effects of UV irradiation include UV-induced erythema. Sunlight plays an important role in the development of skin cancer. Several predictive factors of UV-induced erythema could also be predictive for skin cancer.
Objective: Our objective was to quantitatively assess phenotypical and nutritional determinants of sensitivity to UV-irradiation, as assessed by the minimal erythema dose (MED).
Design: We conducted a cross-sectional study among 335 volunteers. Sensitivity to UV irradiation was established through assessment of the MED. Phenotypical determinants, including skin melanin content, hair color and iris color were determined by skin reflectance spectrometry, a subjective questionnaire and an objective classification system, respectively. Furthermore, dietary exposure was measured by carotenoids, vitamin C, retinol and α-tocopherol in serum.
Results: Male subjects were found to be more sensitive to UV irradiation, i.e. the MED was significantly lower compared to female subjects. Skin melanin content, which was positively associated with iris color in both sexes and with hair color in men, was the main phenotypical determinant of sensitivity to UV-irradiation. No associations were found between serum carotenoids and MED in the total study group. Vitamin C was inversely associated with MED. However, associations between carotenoids concentrations and MED showed a positive trend in subjects with melanin values above and a negative trend in subjects below the median after adjustment for gender and total cholesterol.
Conclusions: Skin melanin content and gender are important determinants of sensitivity to UV-irradiation. No relation was found between serum carotenoids and MED in the total study group. The inverse association between vitamin C and MED was against our hypothesis. For the modifying effect of melanin on the association between carotenoids and MED we do not have a clear biological explanation.

Introduction

The effects of solar irradiation on human health predominantly result from ultraviolet (UV) light absorption by the skin and eyes. An important beneficial effect is the stimulation of production of vitamin D_3 in the skin, which is essential for calcium absorption and bone formation (Bickle and Pillai, 1993). Sun exposure may also stimulate the physical and mental condition of people (Meesters *et al.*, 1990). On the other hand, UV irradiation could also cause damage in light-exposed tissues; in the skin this may lead to sunburn, skin aging, immunosuppression and skin cancer (Lapière, 1990; Brash *et al.*, 1991; Kripke *et al.*, 1992; Vink *et al.*, 1996).

Sunburn is a well-known acute effect of excessive UV exposure. Low dose or short exposure to UV irradiation is tolerated by the skin without noticeable or clinically relevant changes. Only after reaching a certain threshold, a delayed and prolonged vasodilation develops allowing passage of lymphocytes and macrophages into the tissue and inducing an inflammatory response, which is clinically visible as erythema (Luger and Schwarz, 1990; Boelsma *et al.*, 2001).

Erythema is the most clinically apparent component of a sunburn reaction. A frequently used measure for the skin's sensitivity to UV irradiation is the minimal erythema dose (MED). One MED is the minimal amount of energy required to induce visible erythema, which can be defined as a uniform, clearly demarcated or a just perceptible redness at 16–24 h after UV exposure. Thus, a low MED indicates a high sensitivity to sunburn (Boelsma *et al.*, 2001).

Several factors, such as skin type and hair color may be predictive of UV-induced erythema (Azizi *et al.*, 1988). In epidemiological studies on skin cancer it has been found that phenotypical factors, such as eye color, hair color, skin type and skin color are major determinants of skin cancer risk (Lock-Andersen *et al.*, 1999; Bliss *et al.*, 1995; Amstrong and Kricker, 2001).

Besides these phenotypical determinants, nutritional compounds are likely to influence the UV-induced erythema reaction by modulation of the inflammatory response. This may be mediated by diet-derived compounds, capable of scavenging reactive oxygen species (ROS), which are generated during the UV-induced inflammatory response (Bast *et al.*, 1998).

The effects of several nutritional compounds on MED, including carotenoids and vitamins E and A have been studied in small intervention studies. The results of

these studies were, however, inconclusive and contradictory (Stahl *et al.*, 2000; Lee *et al.*, 2000; Gollnick *et al.*, 1996; Garmyn *et al.*, 1995; Wolf *et al.*, 1988; Eberlein-König *et al.*, 1998; Fuchs *et al.*, 1998; La Ruche and Cesarini, 1991; Werninghaus *et al.*, 1994). Moreover, all these studies used synthetic (pure) compounds in relatively high dosages compared to the normal physiological range of vitamins and carotenoids.

The objective of the present study was to quantitatively assess phenotypical and nutritional determinants at a usual exposure level of skin sensitivity to UV-irradiation. To address the objectives the associations between skin color, iris color, hair color and skin sensitivity to UV irradiation were evaluated. Furthermore, we investigated whether carotenoids and vitamins were associated with skin sensitivity to UV irradiation in subjects with normal dietary intake levels.

Subjects and methods

Subjects

The subjects in our study were recruited from the pool of volunteers of TNO Nutrition and Food Research and through advertizing in local and regional newspapers and on television. The cross-sectional data analyzed here were gathered during the baseline measurements of an intervention study designed to test an unrelated hypothesis. Advertizing aimed at recruiting people with a general interest in participating in nutrition studies. Respondents who expressed potential interest received a questionnaire eliciting data on daily fruit and vegetable consumption and lifestyle factors, such as smoking habits and alcohol consumption. This questionnaire was designed for ranking subjects according to their intake and not for quantitative measurements. A total of 2734 out of 6900 questionnaires were returned. Respondents in the highest quintile of fruit and vegetable consumption and those in the lowest tertile (calculated in the total group) were invited for an oral briefing and screening. In the lowest stratum vitamin supplement users were excluded. The major inclusion criteria were an age of 18–75 years and consumption of an average Western diet. The main exclusion criteria were pregnancy and/or lactation or wishing to become pregnant, serum cholesterol > 7.5 and/or triacylglycerol > 2.3 mmol/L if not under stabilized hypercholesterolemia/hyperlipidemia treatment, and anticoagulant therapy.

Three hundred and eighty volunteers entered the study. Informed consent was obtained from all subjects.
The study was performed according to ICH (International Conference on Harmonization of Technical Requirements of Registration of Pharmaceuticals for Human Use) guidelines for good clinical practice, and was approved by an external Medical Ethical Committee. The study was conducted at the Department of Nutritional Physiology of TNO Nutrition and Food Research, Zeist.

UV skin sensitivity test
The minimal erythema dose (MED) of each volunteer was assessed to determine the sensitivity to UV irradiation. Six areas (1 cm^2 each) within a total area of 35 cm^2 on the buttock skin were irradiated with a 150W Xe arced equipped solar simulator (Oriel, Stratford, USA) with incremental doses using 1.25 geometric progression between each successive UV (UVB and UVA) dose. The test sites were free from blemishes and had an even color tone. During exposure the subject was in a sitting and upright position. The following day, i.e. 20 ± 4 hours after the irradiation, the dose resulting in a just perceptible redness was recorded as each volunteer's personal MED (MED).
A PMA2100 dosimeter (Solar Light, Philadelphia, USA) equipped with a biologically weighted UVB sensor (PMA2103) was used to determine the dose of biologically effective UV irradiation. The sensor's spectral response followed closely the erythema action spectrum (McKinlay and Diffey, 1987).
One standard MED (sMED) was the effective dose of irradiation (this is usually not on buttock skin) capable of causing minimum erythema in an average skin type 2. The relationship between sMED/h and radiometric units was as follows: 1 sMED/h = 5.83 $\mu W/cm^2$; i.e., 1 sMED equals 210 J/m^2 at 297 nm.

Determination of skin melanin
Melanin content was measured on the non-exposed buttock skin using a Mexameter (MX16, Courage & Khazaka Electronic GmnH, Cologne, Germany) 1 year after MED determination in 341 volunteers. In this instrument, light was emitted at three defined wavelengths 568 nm (green), 660 nm (red) and 880 nm (infrared). A photodetector measured the light reflected by the skin. A melanin index (brown color) was computed from the intensity of the absorbed and the

reflected light at, respectively, 660 and 880 nm. Melanin indices were expressed as arbitrary units (a.u.).

Determination of iris color

Iris color was graded by a five-grade classification system, performed by one single investigator (Seddon *et al.*, 1990). Briefly, blue and gray irises were classified as grade 1 or 2. These grades were distinguished by assessing the proportion of total iris area with brown or yellow pigment. A green iris was assigned grade 2 or 3. Similarly, these grades depended on the extent of brown or yellow pigment in the iris. A predominantly brown iris was assigned grade 3, 4 or 5, depending on the intensity of the yellow or brown pigment.

Determination of hair color

Subjects were asked to indicate their natural (un-dyed) hair color in accordance with a six-point scale (red, light blond, dark blond, brown, black or gray). If they had gray hair, they had to indicate their original hair color (red, light blond, dark blond, brown, and black). The original hair colors of the volunteers were used in the analyses.

Blood sampling

For the analysis of carotenoids, retinol and α-tocopherol, blood was collected in tubes containing clot activator and gel (Becton Dickinson, Vacutainer systems, Netherlands). These tubes were immediately stored in a closed box, to avoid breakdown of carotenoids by UV irradiation. Tubes were centrifuged within 15–30 min after collection at ca. 2000 × *g* for 10 min at ca. 4°C to obtain serum. After centrifugation serum was removed and stored at ca. –80°C. All serum handling before storage was done in subdued light.

For the analysis of vitamin C, blood was collected in tubes containing lithium heparin (Becton Dickinson, Vacutainer systems). A 0.5 mL aliquot of blood was added to 2 mL metaphosphoric acid (50 g/L; Mallinckrodt Baker, Deventer, Netherlands) before freezing to preserve the vitamin C concentration during storage. This mixture was stored at ca. –80°C.

For the analysis of total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol blood was collected in tubes containing clot activator and gel

(Becton Dickinson, Vacutainer systems). Tubes were centrifuged within 15–30 min after collection at ca. 2000 × *g* for 10 min at ca. 4°C to obtain serum. After centrifugation serum was removed and stored at ca. –20°C.

Analysis of carotenoids, retinol and α-tocopherol in serum

At the day of analysis serum samples were thawed and mixed well. Samples were deproteinized by adding 500 µL sample to 500 µL ethanol (Nedalco, Bergen op Zoom, Netherlands) containing tocol (Matreya, Pleasant Gap, USA) which was used as internal standard. After mixing, the samples were allowed to stand for 15 min at room temperature to complete precipitation of proteins. Subsequently, 1.0 mL *n*-hexane (Merck, Darmstadt, Germany) was added and vortexed for 4 min. After centrifugation (2000 × *g*, 10 min, 4°C), 0.5 mL of the upper hexane layer was evaporated to dryness. The residue was dissolved in 0.5 mL of a mixture of methanol (Rathburn Chemicals, Walkerburn, UK), acetonitrile (Biosolve, Valkenswaard, Netherlands) and dichloromethane (Mallinckrodt Baker, Deventer, Netherlands). A 50 µL portion was injected on the HPLC system. Quality of the measurements was assessed by analyzing plasma quality control samples in each batch. The coefficients of variation in the quality control samples were for the analysis of lutein 3.7% (mean concentration: 0.16 µmol/L), zeaxanthin 11.5% (0.03 µmol/L), β-cryptoxanthin 8.3% (0.11 µmol/L), all-*trans* lycopene 6.2% (0.31 µmol/L), α-carotene 14.9% (0.05 µmol/L), β-carotene 5.8% (0.25 µmol/L), α-tocopherol 2.8% (20.9 µmol/L) and retinol 2.5% (1.6 µmol/L).

The HPLC system consisted of the following equipment: a Waters 2690 solvent delivery/injection system (Waters, Etten-Leur, Netherlands), a Waters 996 diode array and a Jasco 920 fluorescence detector (Jasco Benelux, Maarssen, Netherlands). System control and data acquisition /processing was performed by Millennium^{32} chromatography manager version 3.05 (Waters).

Separation was obtained by two C18 reversed-phase columns (250 mm × 4.6 mm, 5 µm) in series, thermostatically controlled at 30°C. The samples were eluted using a gradient consisting of methanol, acetonitrile, 2-propanol (Mallinckrodt Baker) and milliQ® (Millipore, Etten-Leur, Netherlands) at a flow rate of 1.5 mL/min. This HPLC system allowed simultaneous detection of retinoids, tocopherols and carotenoids as well as their isomers. Additionally, the diode array data provided spectral information for confirmation of the identity of the analytes.

Analysis of vitamin C in blood

Vitamin C was extracted from whole blood using metaphosphoric acid (Mallinckrodt Baker). Samples were allowed to stand for 20 min at 4°C to precipitate the proteins and to oxidize ascorbic acid to dehydroascorbic acid. After centrifugation samples were subsequently derivatized with 1,2-diaminobenzene (Merck) and injected on the HPLC system. In each series blood quality control samples were analyzed to check for reproducibility. The reproducibility of the assay was 10%. HPLC analysis was performed on an isocratic reversed-phase system consisting of a Gilson 234 injector (Meyvis, Bergen op Zoom, Netherlands), a Gynkotek model 300 pump (Separations, Hendrik-Ido-Ambacht, Netherlands), a C18 column (125 mm × 4.6 mm, 5 μm) and a Jasco 920 fluorescence detector. Mobile flow rate was 1.1 mL/min and consisted of methanol and buffer pH 7.8. Data acquisition and processing were performed by Turbochrom version 6.1.2.0.1:D19 (PerkinElmer Instruments, Nieuwerkerk a/d IJssel, Netherlands).

Analysis of serum lipids

Serum triacylglycerols were analyzed by enzymatic hydrolysis with subsequent enzymatic determination of the liberated glycerol by colorimetry (commercial available kit; Boehringer, Mannheim, Germany). Total cholesterol was analyzed by enzymatic conversion to a stable chromogen, which can easily be detected by colorimetry (Boehringer, Mannheim, Germany). HDL cholesterol was analyzed by precipitation with polyethylene glycol, centrifugation and enzymatic detection by colorimetry (Boehringer, Mannheim, Germany) and LDL cholesterol was calculated using the Friedewald formula (Friedewald *et al.*, 1972).

Statistical analyses

The statistical software package SAS/STAT (release 6.12, SAS Institute, Cary, North Carolina, USA) was used for data analysis. For analysis, variables were subdivided in phenotypical determinants (skin melanin content, hair color and iris color), life-style and demographic factors (gender, smoking, age and fruit and vegetable intake) and nutritional determinants (serum carotenoids and vitamins).
Linear regression modeling (General Linear Model (GLM)-procedure) was used to evaluate the associations between MED and these variables separately.

Furthermore, the contribution of nutritional determinants to MED, after adjustment for potential confounders was evaluated starting with the maximum model, including melanin index, total cholesterol, gender and the interaction terms (carotenoids/vitamins × gender) and (carotenoids/vitamins × melanin index).
Because of the significant interaction term β-carotene × melanin, a class variable was created for melanin at the median level (≤ and > 470 a.u.) and the analyses were repeated. The influence of melanin on the associations between carotenoids and MED was not our hypothesis, but a possibly relevant finding to present.
Data of 6 volunteers were not included because 4 volunteers were not irradiated and 2 volunteers showed erythema responses at all irradiation doses. Iris color and hair color were not classified for all participants, so we presented the results of 290 volunteers for hair color of 322 volunteers for iris color. Two volunteers had a very high melanin index value (> 3 SD of the mean). We repeated the analyses after exclusion of the data of these two volunteers. All regression equations were the same.

Results

Study population

We aimed at recruiting a study population with a wide range of fruit and vegetable intake, including fruit juices. In the end, significantly higher serum levels of carotenoids and vitamins were found in the group, selected on high fruit and vegetable intake in comparison with the low fruit and vegetable group. However, in comparison with other countries, for example the United States and South European countries (Olmedilla *et al.*, 2001; Mares-Perlman *et al.*, 2001) the range in serum carotenoids was rather narrow.
The mean MED as was measured in 335 volunteers was 0.56 ± 0.20 × sMED (114.5 J/m^2). Life style and demographic factors of the study population and associations with MED are presented in Table 1. Men displayed approximately 16% lower MED values than women (P = 0.0001). No differences were observed in MED values between age categories (P = 0.40), between smokers and non-smokers (P = 0.86) and between low and high fruit and vegetable consumption categories (P = 0.07).

Table 1 Mean MED values for men and women, smokers and non-smokers, different age categories and high and low fruit and vegetables consumers

Characteristics	*n*	MED[1]
Gender		
Men	161	0.51 ± 0.18[2]
Women	174	0.61 ± 0.20
Smoking		
No	236	0.56 ± 0.20
Yes	99	0.56 ± 0.20
Age		
Low (18–30 y)	85	0.54 ± 0.17
Middle (31–50 y)	152	0.57 ± 0.19
High (51–75 y)	98	0.57 ± 0.23
Fruit and vegetable consumption		
Low	193	0.54 ± 0.18
High	142	0.58 ± 0.22

[1] MED: personal minimal erythema dose, expressed in standard MED (mean ± SD). [2] Significantly different from women; $P = 0.0001$.

Phenotypical determinants

Mean values of MED for the different categories of phenotypical determinants (skin melanin index, hair color and iris color) in men and women are presented in Table 2. Skin melanin index was associated with iris color both in men and women. Hair color was associated with skin melanin index in men only. Of course, these determinants are related to common genetic factors, resulting in interrelations.

Positive associations between hair color, iris color and MED were found in men only. Skin melanin index was associated with MED in both men and women. Hair color and iris color may serve as proxy measurements of skin melanin index. Melanin index was measured in the skin, the target organ of the MED measurement. Therefore, we included skin melanin index in the regression models. The regression coefficient (± SE) of melanin index was 0.36 ± 0.04 per 100 a.u. in the univariate model with an explained variance (R^2) of 17%. In the multivariate model including melanin index and gender, the regression coefficients were 0.39 ± 0.04 per 100 a.u. and –0.12 ± 0.02 (men), respectively (R^2 = 27%). Regression

coefficients of iris color classes or hair color classes in the model including melanin index and gender were not significant, did not contribute to the explained variance and did not affect the regression coefficients of melanin and gender.
Both skin melanin index and gender were important independent predictors of MED and were included in the regression models to further evaluate the influence of nutritional determinants on MED.

Table 2 Mean personal minimal erythema dose (MED) per category of phenotypical determinants in men and women

	Men		Women	
Variable	*N*	MED[1]	*n*	MED[1]
Hair color				
Red	1	0.30	4	0.76 ± 0.20
Light blond	11	0.43 ± 0.07	18	0.59 ± 0.25
Dark blond	78	0.50 ± 0.16	88	0.60 ± 0.20
Brown	30	0.47 ± 0.13	39	0.62 ± 0.22
Black	14	0.67 ± 0.31[2]	7	0.57 ± 0.14
Iris color				
Blue or gray	98	0.48 ± 0.14	113	0.60 ± 0.19
Blue, gray or green	28	0.51 ± 0.23	22	0.62 ± 0.23
Green or light brown	11	0.42 ± 0.11	15	0.59 ± 0.24
Brown	13	0.61 ± 0.17	12	0.62 ± 0.21
Dark brown	6	0.78 ± 0.39[3]	4	0.73 ± 0.25
Skin melanin index				
≤ 470 a.u.	72	0.46 ± 0.14	97	0.52 ± 0.20
> 470 a.u.	89	0.55 ± 0.20[2]	77	0.70 ± 0.20[3]

[1] MED: personal minimal erythema dose, expressed in standard MED. [2] Variable associated with MED in linear regression modeling; $P < 0.01$. [3] Variable associated with MED in linear regression modeling; $P < 0.001$.

Nutritional determinants

Mean concentrations of serum carotenoids and vitamins and regression coefficients in relation to MED are presented in Table 3. No significant associations between MED and serum carotenoids or vitamins A and E after adjustment for total

cholesterol and gender were found in the total study group. Only vitamin C was inverse associated with MED after adjustment for gender.

The contribution of serum carotenoids and vitamins separately on MED were evaluated after adjustment for melanin index, gender and total cholesterol. Firstly, the model with the independent variables gender and total cholesterol and the interaction terms (carotenoids/vitamins × gender) and (carotenoids/vitamins × melanin index). The regression coefficients (β) of the interaction term gender × carotenoids was not significant for the different carotenoids, except for α-carotene The interaction term carotenoids/vitamins × melanin index was significant for β-carotene.

Because of the significant interaction term β-carotene × melanin, a class variable was created for melanin at the median level (≤ and > 470 a.u.).

In the model with the class variable for melanin, significant interaction terms were found between serum lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and the class variable for melanin. The interaction terms β-carotene × gender and α-carotene × gender were significant.

Table 3 shows the regression coefficients of the model with the variables gender and total cholesterol of the carotenoids and vitamins in subjects with high and low melanin values. Positive trends were observed for subjects with high skin melanin values and negative trends for subjects with low skin melanin values. The regression coefficients in the low skin melanin group for α-carotene, β-carotene were –0.032 ± 0.031 ($P = 0.31$) and 0.006 ± 0.007 ($P = 0.41$) in women and –0.057 ± 0.036 ($P = 0.11$) and –0.018 ± 0.009 ($P = 0.04$) in men, respectively. In the high skin melanin group the regression coefficients for α-carotene, β-carotene were 0.129 ± 0.035 ($P = 0.0004$) and 0.026 ± 0.011 ($P = 0.02$) in women and –0.034 ± 0.030 ($P = 0.26$) and –0.0005 ± 0.011 ($P = 0.96$) in men, respectively.

Table 3 Serum carotenoids and vitamins concentrations in 335 volunteers and regression coefficients in relation to minimal erythema dose (MED) after adjustment for gender and total cholesterol in the total group and in the high and low melanin group

	Serum mean ± SD µmol/L	Total group β ± SE[3] *P*-value	Low melanin[1] β ± SE[3] *P*-value	High melanin[2] β ± SE[3] *P*-value
Lutein	0.18 ± 0.08	0.005 ± 0.015	−0.033 ± 0.017	0.044 ± 0.021
		0.72	0.06	0.04
Zeaxanthin	0.05 ± 0.02	−0.014 ± 0.054	−0.12 ± 0.062	0.11 ± 0.080
		0.79	0.055	0.17
β-Cryptoxanthin	0.20 ± 0.12	0.005 ± 0.009	−0.016 ± 0.011	0.023 ± 0.013
		0.59	0.15	0.08
Lycopene	0.36 ± 0.18	0.006 ± 0.006	−0.004 ± 0.007	0.007 ± 0.008
		0.30	0.56	0.43
α-Carotene	0.07 ± 0.06	0.011 ± 0.017	−0.041 ± 0.023	0.031 ± 0.023
		0.55	0.08	0.18
β-Carotene	0.37 ± 0.22	0.006 ± 0.005	−0.002 ± 0.006	0.011 ± 0.008
		0.24	0.75	0.12
α-Tocopherol	26.8 ± 6.1	0.001 ± 0.002	−0.002 ± 0.003	0.003 ± 0.003
		0.74	0.49	0.41
Retinol	2.04 ± 0.45	−0.01 ± 0.02	0.008 ± 0.027	−0.029 ± 0.037
		0.68	0.77	0.43
Vitamin C	45 ± 14	−0.002 ± 0.001	−0.001 ± 0.001	−0.002 ± 0.001
		0.04	0.16	0.15

[1] n = 169. [2] n = 166. [3] regression coefficient and standard error per 0.1 µmol/L for carotenoids and per 1 µmol/L for vitamins; models are adjusted for gender and total cholesterol. Model for vitamin C is adjusted for gender.

The interactions between melanin and carotenoids could be influenced by gender differences in skin melanin content, but after exclusion of gender from the model, the product terms melanin × carotenoids did not change. Therefore, we concluded that this interaction could not be explained by the difference in the gender distribution in skin melanin index. The regression coefficient of the class variable for melanin changed more than 10% after exclusion of gender. This could implicate confounding by gender.

The differences in MED values between men and women could be influenced by different serum concentrations of carotenoids. However, the regression coefficient (± SE) of gender in the multivariate model with gender, total cholesterol and carotenoids, separately was –0.10 ± 0.02. The regression coefficient of gender was the same as the regression coefficient in the bivariate model with only gender and total cholesterol as independent variables (β = –0.10, SE = 0.02). This means that difference between men and women in MED values cannot be explained by differences in serum carotenoid concentrations.

In summary, the main phenotypical determinant of UV-sensitivity was skin melanin and men were more sensitive to UV-irradiation than women. Associations between serum carotenoids and MED showed a positive trend in subjects with melanin values above and a negative trend in subjects below the median after adjustment for gender and total cholesterol. For serum α-carotene and β-carotene, this pattern was only seen in women.

Discussion

The mean MED in this general population with a 'normal' dietary intake was 0.56 ± 0.20 × sMED. Men had a lower MED value than women, which reflects higher sensitivity to UV-irradiation. Skin melanin content was the main predictor of the MED in our study. Vitamin C was inversely associated with MED against our hypothesis. Serum lutein, zeaxanthin, β-cryptoxanthin and lycopene concentrations were positively associated with MED after adjustment for gender and total cholesterol in subjects with high skin melanin and negatively associated in subjects with low skin melanin values. For serum α-carotene and β-carotene, this pattern was only seen in women.

These results are based on a large study group with a normal dietary intake. The serum concentrations of carotenoids and vitamins could reflect the usual dietary intake. There were several limitations of this study that may attenuate the results of this study. We selected Dutch volunteers with high and low fruit and vegetable consumption in order to aim a broad range in carotenoid exposure. The absolute range in serum carotenoids is narrow in comparison with serum carotenoid concentrations in the United Stated and South European countries (Olmedilla *et al.*, 2001; Mares-Perlman *et al.*, 2001). The evaluation of a large number of associations could cause that significant results are chance findings. Previous

intervention studies showed both positive effects of supplementation of either β-carotene or a combination of carotenoids on UV sensitivity (Stahl *et al.*, 2000; Lee *et al.*, 2000; Gollnick *et al.*, 1996) or no effect on UV sensitivity (Garmyn *et al.*, 1995; Wolf *et al.*, 1988). The duration of the supplementation might be an important factor. In the studies, which showed a protective effect, the duration of the study was more than 10 weeks. All of these supplementation studies used a very high dosage of carotenoids and selected the volunteers on skin type. However, in a cross-sectional design it is difficult to assess the temporal relationship between nutritional factors, for example carotenoids and the dependent variable, in this case minimal erythema dose.

Evaluation of the association between carotenoids and vitamins within a normal physiological range and MED were based on concentrations of these substances in serum but not in the target tissue skin. However, it was previously shown that plasma and skin concentrations of carotenoids and α-tocopherol showed significant correlations, suggesting that the status of these micronutrients in skin may be estimated from the plasma concentrations (Peng *et al.*, 1993; Peng *et al.*, 1995; Stahl *et al.*, 1998).

The mean MED (0.56 × sMED) being below one sMED, may indicate that: 1) the Dutch subjects recruited in this study are on average more sensitive than a phototype II, or, more likely, 2) the buttock skin used for the MED test is more sensitive than the skin usually used for such a test.

In our study, men had a lower MED value than women, which reflects higher sensitivity to UV-irradiation in men. Several factors may account for this difference. One factor could be a difference in skin melanin index between men and women, suggesting that subjects with higher melanin content are less sensitive to UV-light and have therefore higher MED values. Skin melanin content was significantly higher in men than in women in this study, 478 ± 26 and 472 ± 19 (P = 0.03), respectively which seemed to be in contrast to the lower MED observed in men. However, after exclusion of two male volunteers with a high skin melanin content, there was no difference in skin melanin content between men and women, 476 ± 20 and 472 ± 19 (P = 0.08) respectively. This exclusion did not affect the significant higher MED values in women in comparison with men. Skin melanin content was the main predictor of the MED in our study. Melanin is a

photoprotective agent in the target tissue skin and therefore protects against the effects of UV irradiation (Riley, 1997).

In addition to melanin, antioxidants in skin (carotenoids and vitamins) may play an important role in skin protection against UV irradiation. The possible protective mechanisms are reflection, reaction with active oxygen species and quenching of radicals (Bast *et al.*, 1998).

Associations between serum carotenoids concentrations and UV-sensitivity after adjustment for gender and total cholesterol were dependent on a theshold value of melanin. The influence of the serum carotenoids on UV sensitivity was not concealed in the gender effect, because the regression coefficient of gender did not change after inclusion of serum carotenoids, separately.

We do not have a clear biological explanation for the finding of a threshold value for skin melanin content on the association between serum carotenoids and MED. Although a role of melanin was not hypothesized in the association between carotenoids and MED, it is a possibly relevant finding to present. The combination of high melanin concentrations and carotenoids could be an important mechanism, especially where the maintenance of the carotenoid concentrations is vital to avoid deleterious effects of UV light, such as in the retina of the eye and the skin (Edge *et al.*, 2000). Therefore, it could not be excluded that melanin content might have an influence on the association between carotenoids and MED. However, the role of melanin on the association between serum carotenoids and MED could also be a chance finding.

All carotenoids are present in the skin, including lycopene with very high concentrations in the skin. Concentrations of β-carotene and lycopene in the skin are approximately 216 ± 97 and 126 ± 90 ng/g wet tissue in non-smokers (Peng *et al.*, 1995). We could not confirm the strong positive influence of lycopene on erythema according to a nutritional supplementation study (Stahl *et al.*, 2001). They reported a protection against UV light-induced erythema in humans after consumption of tomato paste (approximately 16 mg/d lycopene).

Although the efficacy of protection is not comparable to the protection factor of sunscreens, dietary intake of carotenoids could increase the basal protection of skin against erythema, suggested by others (Stahl *et al.*, 2001). In our study, this basal protection was only attributable to subjects with high skin melanin content. More research to investigate the influence of nutritional compounds on sensitivity to UV-

light is necessary. Also the possible influence of skin melanin on the association between nutritional compounds and sensitivity to UV-light has to be studied.
From our results we conclude that men are more susceptible to UV irradiation than women. No associations between serum carotenoids and MED were found in the total study group. However, this association was modified by skin melanin content, although we do not a have a clear biological explanation for this. Serum carotenoid concentrations in a normal physiological range showed a positive trend with UV sensitivity in subjects with high melanin index and negative trend in subjects with low melanin index values. Dietary intervention studies are necessary to study the influence of nutritional compounds on the skin sensitivity to solar irradiation in both men and women and the possible interaction between nutritional compounds and melanin.

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Chapter 5

Serum carotenoids and vitamins in relation to biomarkers of endothelial function and inflammation

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Abstract

Background: The vascular endothelium is the primary site of dysfunction in atherosclerosis. Endothelial cell dysfunction may be related to an increase in cellular oxidative stress. Carotenoids and vitamins could have an antioxidant mediated tempering influence on endothelial function and inflammation, thereby reducing the risk of atherosclerosis. The objective of this study was to test the hypothesis that serum carotenoids and vitamins are associated with markers of endothelial function and inflammation.

Methods: We measured serum carotenoids, α-tocopherol and vitamin C concentrations in 379 subjects sampled from the general population. High-sensitive C-reactive protein (CRP), fibrinogen (Fbg) and leucocytes were measured as markers of inflammation. Furthermore, soluble intercellular adhesion molecule-1 (sICAM-1) and flow-mediated vasodilation (FMD) were measured as markers of endothelial function; the latter in a subset of 165 subjects. Relationships between serum carotenoids and vitamins and markers of endothelial function and inflammation were analyzed after adjustment for confounding, notably age, gender, body mass index (BMI), total cholesterol and smoking status (current vs non-current).

Results: Most of the carotenoids and markers of endothelial function and inflammation were associated with age, gender, smoking status, BMI and total cholesterol. In the total study group, lutein and lycopene were inversely related to sICAM-1 with regression-coefficients of -0.38 ± 0.19 ($P = 0.04$) and -0.16 ± 0.08 ($P = 0.04$) per 1 μmol/L, respectively. β-Carotene was inverse related to leucocytes (-0.23 ± 0.07; $P = 0.007$) and CRP (-1.09 ± 0.30; $P = 0.0003$) per 1 μmol/L. Vitamin C was inverse related to CRP (-0.01 ± 0.005; $P = 0.04$) per 1 μmol/L, whereas α-tocopherol was positively related to CRP (0.03 ± 0.01; $P = 0.02$) per 1 μmol/L. Zeaxanthin was inversely related to FMD (31.2 ± 15.3; $P = 0.04$) per 1 μmol/L. No significant relations associations were found between carotenoids, vitamins and fibrinogen.

Conclusion: The inverse relations between carotenoids, vitamin C and sICAM-1, CRP and leucocytes may help to explain the possibly protective effect of carotenoids and vitamin C on atherosclerosis through an influence on inflammatory processes and endothelial function.

Introduction

The vascular endothelium is the primary site of dysfunction in atherosclerosis. The endothelium is an active and dynamic organ and is essential in the processess of cell adhesion and migration, thrombosis, and fibrinolysis (Brown and Hu, 2001). In humans the endothelium is continuously exposed to pro-oxidative and anti-oxidative factors, which are normally in balance (Hennig and Toborek, 2001). Endothelial cell dysfunction may be related to an increase in cellular oxidative stress, an imbalance between pro- and anti-oxidative factors. Oxidative stress can activate transcription factors, which promote cytokine production, expression of adhesion molecules and acute phase molecules and thus intensify an inflammatory response in atherosclerosis (Navab *et al.*, 1991; Marui *et al.*, 1993; Berliner *et al.*, 1995; Ross, 1999). In simple terms, a high oxidative stress may be related to endothelial dysfunction, elevated levels of adhesion molecules and elevated levels of inflammatory factors.

There is growing interest in the role of antioxidants in preventing oxidative stress and modulating endothelial cell function, thereby reducing the risk of vascular dysfunction. Evidence to support the importance is based on an inverse association between low carotenoid concentrations and risk of cardiovascular disease (Gey *et al.*, 1993, Kardinaal *et al.*, 1993, Street *et al.*, 1994). In addition, inverse associations have been found between β-carotene and inflammation in patients with inflammatory diseases (Curran *et al.*, 2000) and healthy subjects (Erlinger *et al.*, 2001).

Dynamic endothelial function can be assessed by measurement of flow-mediated vasodilation (FMD). Impaired FMD has been related to established risk factors, but the predictive value has not been established (Celermajer *et al.*, 1994). There is overwhelming evidence to link elevated adhesion molecules, such as soluble intercellular adhesion molecule-1 (sICAM-1) to unfavorable levels of cardiovascular disease risk factors such as smoking, blood pressure, HDL and total cholesterol (Demerath *et al.*, 2001). Also sICAM-1 has been shown to predict cardiovascular disease in some studies (Ridker *et al.*, 2000; Blankenberg *et al.*, 2001). Several inflammation markers, including C-reactive protein (CRP), fibrinogen (Fbg) and leucocyte count, have consistently been shown to predict cardiovascular heart disease (Danesh *et al.*, 1998; Tracy *et al.*, 1999, Ridker *et al.*, 2000). Because of previous findings, we hypothesize that antioxidants are

associated with endothelial function and inflammation processes. To test this hypothesis we evaluated the association between serum antioxidants (carotenoids and vitamins) and markers of endothelial function (sICAM-1 and FMD) and inflammation (CRP, Fbg, leucocytes).

Methods

Study population

The 380 subjects in our study were recruited from the pool of volunteers of TNO Nutrition and Food Research and through advertizing in local and regional newspapers and on television. The cross-sectional data analyzed here were obtained during baseline measurements for a dietary intervention study designed to test an unrelated hypothesis. Advertizing aimed at recruiting people who had a general interest in participating in nutrition studies. Subjects who expressed potential interest received a questionnaire on daily fruit and vegetable consumption and lifestyle factors. A total of 2734 out of 6900 questionnaires were returned. Subjects in the highest quintile of fruit and vegetable consumption and those in the lowest tertile (calculated in the total group) were invited for an oral briefing. In the lowest stratum, vitamin supplement users were excluded. After the oral briefing, 589 subjects were interested to participate and were screened.

The major inclusion criterium was an age between 18–75 years and consumption of an average Western diet. The main exclusion criteria were pregnancy and/or lactation or wishing to become pregnant; cholesterol > 7.5 mmol/L and/or triacylglycerol > 2.3 mmol/L if not under stabilized hypercholesterolemia /hyperlipidemia treatment, and anticoagulant therapy. A total of 413 volunteers were allocated to an entry number. Before the start of the study, 33 subjects withdrew from the study. At day one, 380 volunteers (179 men, 201 women) entered the study. Written informed consent was obtained from all subjects.

The study was performed according to ICH (International Conference on Harmonization of Technical Requirements of Registration of Pharmaceuticals for Human Use) guidelines for good clinical practice, and was approved by an external Medical Ethical Committee. The study was executed at the Department of Nutritional Physiology of TNO Nutrition and Food Research, Zeist, and the measurements were performed from June 29 to September 16, 1998.

The final study group comprised 178 men and 201 women (pre and postmenopausal). Of these volunteers 165 volunteers (85 men, 80 women) underwent a flow-mediated vasodilation measurement.

Blood sampling

Blood samples were obtained between 800 and 930h after an overnight fast. For the analysis of carotenoids and α-tocopherol, total cholesterol, HDL cholesterol, triacylglycerol, C-reactive protein, blood was collected in tubes containing clot activator and gel (Becton Dickinson, Vacutainer systems). These tubes were immediately stored in a closed box, to avoid breakdown of carotenoids by UV light. Tubes were centrifuged within 15–30 min after collection at ca. 2000 × *g* for 10 min at ca. 4°C to obtain serum. After centrifugation serum was removed and stored at –80°C. All serum handling before storage was done in subdued light. For the analysis of vitamin C and sICAM-1, blood was collected in tubes containing lithium heparin (Becton Dickinson, Vacutainer systems). A 0.5 mL aliquot of blood was added to 2 mL metaphosphoric acid (50 g/L; Mallinckrodt Baker, Deventer, Netherlands) before freezing to preserve the vitamin C concentration during storage. This mixture was stored at –80°C. Thereafter, tubes were centrifuged within 30 min after collection at ca. 2000 × *g* for 20 min at ca. 4°C to obtain plasma. After centrifugation plasma was removed and stored at –80°C. For the analyses of leucocytes, blood was collected in tubes containing K_3EDTA. About 0.5 mL blood at room temperature was used for determination of leucocytes.

Chemical analyses

Serum carotenoids (CVs 3.7–14.9%), retinol (CV 2.5%), α-tocopherol (CV 2.8%) and vitamin C (CV 10%) were quantified by reversed phase HPLC (Broekmans *et al.*, in press). Serum triacylglycerols were analyzed by enzymatic hydrolysis and subsequent enzymatic determination of the liberated glycerol by colorimetry (commercial available kit; Boehringer, Mannheim, Germany). Total cholesterol was analyzed by enzymatic conversion to a stable chromogen, which can easily be detected by colorimetry (Boehringer, Mannheim, Germany). HDL cholesterol was analyzed by precipitation with polyethylene glycol, centrifugation and enzymatic detection by colorimetry (Boehringer, Mannheim, Germany) and LDL cholesterol was calculated using the Friedewald formula (Friedewald *et al.*, 1972). Fibrinogen

was measured according to Clauss (1957) using the STA Fibrinogen kit on a STA analyzer. CRP was analyzed in plasma by an enzyme-immunoassay using polyclonal antibodies (Dako, Copenhagen, Denmark) with a low detection limit to detect basal levels in serum. (De Maat *et al.*, 1996). Soluble ICAM-1 in plasma was determined with an immunoenzymometric method (Gearing *et al.*, 1992) in plasma. The leucocyte count was measured using the Sysmex K-1000.

Flow-mediated vasodilation measurement

Flow-mediated vasodilation (FMD) of the brachial artery was measured by a method described by de Roos *et al.* (2001). In short, the diameter of the artery at rest and at maximum vasodilation was assessed and the percentage increase from baseline was calculated. All measurements were done at end diastole (images were triggered on the use of the R-wave of the electrocardiogram). The ultrasound images were obtained by one technician with a 7.5 MHz linear array transducer of an Ultramark^{tm} 9HDI duplex scanner. All images were stored on super-VHS videotapes for off-line analysis. Off-line readings were performed as described elsewhere (De Roos *et al.*, 2001). In short, the ultrasound interfaces were traced manually of each B-mode image that was stored each 15 seconds after ischemia for a duration of 5 minutes.

Other measurements

Body weight was assessed by weighing the subject wearing indoor clothing, without shoes, wallet and keys. Height was measured without shoes. Body mass index (BMI) was calculated with the formula body weight (kg)/ body height squared (m^2). Smoking status was obtained by a questionnaire; non-smoking and smoking. The use of contraceptives was registered.

Statistical analyses

The SAS statistical software package SAS/STAT (Version 6, SAS Institute, Cary, NC, USA) was used for data analysis. The influence of potential confounders of the relation between carotenoids/vitamins and markers of inflammation and endothelial function was evaluated in univariate linear regression models. Variables that were not normally distributed were natural log-transformed (CRP, sICAM-1 and leucocytes).

Multiple linear regression (GLM procedure) was used to evaluate the associations between carotenoids/vitamins and markers of endothelial function (sICAM-1 and FMD) and markers of inflammation (CRP, Fbg and leucocytes) after adjustment for potential confounding.

Interactions by smoking status, gender and carotenoids or vitamins were evaluated in a general linear model with continuous values of carotenoids and multiplicative interaction terms. If the *P*-value was less than 0.05, the regression coefficients of the variables were considered as significant.

Results

General characteristics of subjects

Table 1 shows the general characteristics of the 379 subjects, 178 men and 201 women.

Table 1 General characteristics of study group[1]

	Men (n = 178)	Women (n = 201)	Total (n = 379)
Smoking (%)	33	27	30
Age (y)	42 ± 15	41 ± 13	42 ± 14
BMI (kg/m^2)[2]	24.0 (20.2 – 28.4)	24.2 (19.9 – 30.9)	24.1 (20.0 – 29.7)
Total cholesterol (mmol/L)	5.4 ± 1.0	5.7 ± 0.9	5.5 ± 1.0
LDL (mmol/L)	3.5 ± 0.9	3.4 ± 0.9	3.5 ± 0.9
HDL (mmol/L)	1.4 ± 0.3	1.8 ± 0.4	1.6 ± 0.4
Triacylglycerols (mmol/L)[2]	1.07 (0.63 – 1.87)	0.98 (0.57 – 1.89)	1.01 (0.59 – 1.88)
Contraceptives	n.a.	34%	n.a.

[1] Mean ± SD unless stated otherwise. [2] Median (10^{th} – 90^{th} percentile). n.a.: not applicable.

Table 2 shows the concentrations of carotenoids, vitamin C and α-tocopherol in men, women and the total group. Table 3 shows the mean (± SD) or median (10^{th} - 90^{th} percentile) values in the total group and in men and women for FMD, sICAM-1, CRP, Fbg, and leucocytes. One hundred and sixty-five subjects, randomly selected, underwent a flow-mediated vasodilation measurement.

Table 2 Serum carotenoid, vitamin C and α-tocopherol concentrations (μmol/L) for men, women and total study group[1]

	Men (n = 178)	Women (n = 201)	Total (n = 379)
Lutein	0.17 ± 0.07	0.19 ± 0.08	0.18 ± 0.08
Zeaxanthin	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02
β-Cryptoxanthin	0.17 ± 0.09	0.23 ± 0.13	0.20 ± 0.12
Lycopene	0.35 ± 0.18	0.37 ± 0.18	0.36 ± 0.18
α-Carotene	0.06 ± 0.06	0.08 ± 0.06	0.07 ± 0.06
β-Carotene	0.33 ± 0.19	0.41 ± 0.25	0.37 ± 0.23
Vitamin C	40.1 ± 12.3	50.0 ± 13.4	45.3 ± 13.8
α-Tocopherol	26.0 ± 5.5	27.7 ± 6.6	27.0 ± 6.2

[1] Mean ± SD.

Table 3 Flow-mediated vasodilation (FMD) values and concentrations of soluble intercellular adhesion molecule (sICAM-1), C-reactive protein (CRP), fibrinogen (Fbg), leukocytes in men, women and total group[1]

	Men (n = 178)	Women (n = 201)	Total (n = 379)
sICAM-1 (ng/mL)[2]	225.5 (175 – 344)	209 (165 – 323)	215 (169 – 328)
FMD (%)	3.9 ± 3.9 (n = 85)	5.1 ± 3.7 (n = 80)	4.5 ± 3.9 (n = 165)
CRP (mg/L)[2]	0.9 (0.2 – 5.9)	1.3 (0.2 – 7)	1.1 (0.2 – 6.7)
Fibrinogen (g/L)	3.2 ± 0.7	3.3 ± 0.6	3.2 ± 0.6
Leucocytes (10^9/L)[2]	5.5 (3.9 – 7.5)	5.4 (3.8 – 8.7)	5.5 (3.9 – 8.1)

[1] Mean ± SD unless stated otherwise. [2] Median (10^{th} – 90^{th} percentile).

Potential confounders

In the total study group, age was positively related to serum lutein, α-carotene, β-carotene and α-tocopherol. Age was inverse related to serum lycopene and was not related to zeaxanthin, β-cryptoxanthin and vitamin C. Women showed higher serum carotenoids than men. Non-smokers showed higher concentrations of carotenoids than smokers. Total cholesterol was positively associated with all carotenoids and α-tocopherol. BMI was inversely related to lutein and β-carotene and positively related to α-tocopherol. Contraceptive use was inversely related to serum β-carotene, α-carotene and lycopene in women.

In the total study group, age was positively related to concentrations of CRP and Fbg. Women showed higher concentrations of CRP and Fbg than men. FMD was lower in men than in women ($P = 0.053$). Smokers showed significantly higher concentrations of Fbg, leucocytes and sICAM-1 than non-smokers. Positive relations were found between total cholesterol and CRP, Fbg and sICAM-1. BMI was positively related to CRP, fibrinogen, leucocytes and sICAM-1. In women, contraceptive use was positively related to CRP and leucocytes.

Multivariate analyses

Age-adjusted and multivariate models adjusted for age, gender, smoking, total cholesterol and body mass index with sICAM-1 and FMD as dependent variables are presented in Table 4. Vitamin C was not adjusted for total cholesterol. Significant inverse relations were found between lutein, lycopene and sICAM-1 concentrations after adjustment. Other carotenoids showed a negative trend with sICAM-1. Only, zeaxanthin was positively related to FMD after adjustment.

Age- and multivariate adjusted models for CRP, fibrinogen and leucocytes are presented in Table 5. After adjustment for age, gender, smoking status, BMI and total cholesterol, β-carotene was inversely and α-tocopherol was positively related to CRP. Vitamin C was inverse related to CRP. None of the carotenoids/vitamins was related to fibrinogen. Only β-carotene was inversely related to leucocytes after adjustment in the multivariate models.

Table 4 Regression coefficients ± SE (*P*-value) of serum carotenoids and vitamins by markers of endothelial function per 1 μmol/L[1]

	sICAM-1[2]	FMD
Lutein		
Age adjusted	−0.57 ± 0.19 (*P* = 0.003)	2.08 ± 4.88 (*P* = 0.67)
Multivariate	−0.38 ± 0.19 (*P* = 0.04)	1.94 ± 5.14 (*P* = 0.71)
Zeaxanthin		
Age adjusted	−1.23 ± 0.68 (*P* = 0.07)	27.4 ± 14.8 (*P* = 0.07)
Multivariate	−0.72 ± 0.66 (*P* = 0.28)	31.2 ± 15.3 (*P* = 0.04)
β-Cryptoxanthin		
Age adjusted	−0.29 ± 0.12 (*P* = 0.01)	4.46 ± 2.44 (*P* = 0.07)
Multivariate	−0.15 ± 0.12 (*P* = 0.22)	3.20 ± 2.61 (*P* = 0.22)
Lycopene		
Age adjusted	−0.11 ± 0.08 (*P* = 0.18)	−3.12 ± 1.64 (*P* = 0.06)
Multivariate	−0.16 ± 0.08 (*P* = 0.04)	−2.63 ± 1.71 (*P* = 0.13)
α-Carotene		
Age adjusted	−0.48 ± 0.23 (*P* = 0.04)	−6.10 ± 5.40 (*P* = 0.26)
Multivariate	−0.31 ± 0.22 (*P* = 0.16)	−8.74 ± 5.70 (*P* = 0.13)
β-Carotene		
Age adjusted	−0.15 ± 0.06 (*P* = 0.02)	−0.57 ± 1.44 (*P* = 0.69)
Multivariate	−0.08 ± 0.06 (*P* = 0.19)	−1.95 ± 1.57 (*P* = 0.22)
Vitamin C[3]		
Age adjusted	−0.003 ± 0.001 (*P* = 0.006)	0.03 ± 0.02 (*P* = 0.27)
Multivariate	−0.001 ± 0.001 (*P* = 0.28)	0.009 ± 0.02 (*P* = 0.70)
α-Tocopherol		
Age adjusted	0.004 ± 0.003 (*P* = 0.14)	0.005 ± 0.06 (*P* = 0.92)
Multivariate	0 ± 0.003 (*P* = 0.99)	0.05 ± 0.07 (*P* = 0.51)

[1] Multivariate models: adjusted for age, gender, smoking, total cholesterol and BMI unless stated otherwise. [2] Log transformed. [3] Multivariate model: adjusted for age, gender, smoking and BMI.

Table 5 Regression coefficients ± SE (*P*-value) of serum carotenoids and vitamins by markers of inflammation per 1 μmol/L[1]

	CRP[2]	Fbg	Leucocytes[2]
Lutein			
Age adjusted	−1.89±0.91(*P*=0.04)	−0.67±0.43(*P*=0.12)	−0.33±0.20(*P*=0.10)
Multivariate	−0.95±0.90(*P*=0.29)	−0.36±0.44(*P*=0.41)	−0.08±0.20(*P*=0.68)
Zeaxanthin			
Age adjusted	−2.31±3.30(*P*=0.48)	0.20±1.55(*P*=0.90)	0.04±0.72(*P*=0.95)
Multivariate	−1.42±3.21(*P*=0.66)	0.80±1.56(*P*=0.61)	0.77±0.71(*P*=0.28)
β-Cryptoxanthin			
Age adjusted	−0.65±0.59(*P*=0.27)	0.03±0.27(*P*=0.91)	−0.29±0.13(*P*=0.03)
Multivariate	−0.58±0.58(*P*=0.31)	0.16±0.28(*P*=0.57)	−0.14±0.13(*P*=0.27)
Lycopene			
Age adjusted	−0.28±0.38(*P*=0.46)	−0.13±0.18(*P*=0.48)	−0.002±0.08(*P*=0.98)
Multivariate	−0.42±0.40(*P*=0.25)	−0.19±0.18(*P*=0.29)	0.003±0.08(*P*=0.97)
α-Carotene			
Age adjusted	−1.67±1.13(*P*=0.13)	−0.37±0.53(*P*=0.48)	−0.50±0.25(*P*=0.047)
Multivariate	−1.07±1.08(*P*=0.32)	−0.11±0.52(*P*=0.83)	−0.28±0.24(*P*=0.25)
β-Carotene			
Age adjusted	−1.33±0.30(*P*=0.001)	−0.23±0.15(*P*=0.11)	−0.32±0.07(*P*=0.001)
Multivariate	−1.09±0.30(*P*=0.0003)	−0.08±0.15(*P*=0.58)	−0.23±0.07(*P*=0.007)
Vitamin C[3]			
Age adjusted	−0.009±0.005(*P*=0.08)	0.0004±0.002(*P*=0.87)	−0.002±0.001(*P*=0.05)
Multivariate	−0.01±0.005(*P*=0.04)	0.0008±0.002(*P*=0.74)	−0.0009±0.001(*P*=0.40)
α-Tocopherol			
Age adjusted	0.05±0.01(*P*=0.0001)	0.01±0.01(*P*=0.08)	−0.004±0.003(*P*=0.16)
Multivariate	0.03±0.01(*P*=0.02)	−0.0005±0.007(*P*=0.95)	0.0003±0.003(*P*=0.93)

[1] Multivariate models: adjusted for age, gender, smoking, total cholesterol and BMI unless stated otherwise. [2] Log transformed. [3] Multivariate model: adjusted for age, gender, smoking and BMI.

A significant interaction between serum α-carotene and smoking status in the model with leucocytes as dependent variable was found. Stratification showed that there was an inverse trend between α-carotene and leucocytes in smokers with a

regression coefficient of −2.23 ± 0.80 (P = 0.006) per μmol/L and no relation in non-smokers (0.004 ± 0.23; P = 0.99). There were significant interactions between α-tocopherol, β-carotene and lycopene and gender in the model with CRP as dependent variable. Stratification showed that there was a positive relation between α-tocopherol and CRP in men (regression coefficient ± SE: 0.08 ± 0.02 per μmol/L; P = 0.001) and no relation in women (0.02 ± 0.02 per μmol/L; P = 0.34). An inverse relation between lycopene and CRP (−1.14 ± 0.54 per μmol/L; P =0.04) was found in men and not in women (0.50 ± 0.50 per μmol/L; P = 0.32). After adjustment for contraceptive use the relation between lycopene and CRP in women was 0.08 ± 0.44 per μmol/L (P = 0.85). β-Carotene was inversely related to CRP (−1.38 ± 0.39 per μmol/L; P = 0.0003) in women and not related in men (−0.19 ± 0.51; P = 0.71). However, after adjustment for contraceptive use in women, the relation was attenuated (−0.58 ± 0.35 per μmol/L; P = 0.10).

FMD values were inversely associated with CRP (regression coefficient ± SE: −1.8 ± 0.7 per 10 mg/L; P = 0.02), sICAM-1 (−0.13 ± 0.04 per 10 ng/mL; P < 0.01) and fibrinogen (−7.8 ± 4.6 per 10 g/L; P = 0.06). Adjusted for age, gender, smoking, BMI and total cholesterol the regression coefficients for CRP, sICAM-1 and fibrinogen were −1.9 ± 0.7 per 10 mg/L (P < 0.01), −0.14 ± 0.04 per 10 ng/mL (P < 0.01) and −7.2 ± 4.5 per 10 g/L (P > 0.05), respectively.

Discussion

We evaluated in a large population based study group the associations between serum carotenoids and vitamins and markers of endothelial function and inflammation. The inverse relations between carotenoids, vitamin C and sICAM-1, CRP and leucocytes may help to explain the possibly protective effect of carotenoids and vitamin C on atherosclerosis through an influence on inflammatory processes and endothelial function.

There were several limitations of this study. Firstly, fruit and vegetables contain multiple agents that could convey benefit to humans, for example fiber, folic acid and polyphenols. In our study, serum carotenoids and vitamins could be a reflection of other compounds in the blood, that are the real substances or variables related to the markers of cardiovascular disease risk. Secondly, residual confounding occurs if confounders are not or insufficiently accounted for in the analysis. Insufficient control for confounders can occur as a result of

misclassification of the confounding factors. Smoking is an important extraneous factor that is associated with both the dependent variables (markers of inflammation and endothelial function) and independent variables (carotenoids). In our models, we included smoking as yes or no and we had no information on ex-smokers, duration of smoking or pack-years smoking. Therefore, we cannot exclude the possibility that the effects could be caused by residual confounding of smoking (Rothman, 1998). Thirdly, the evaluation of a large number of associations could cause that significant results are possibly due to chance.

Dietary antioxidants may protect against oxidative stress by scavenging free radicals and by inhibiting the activation of oxidant-sensitive transcription factors, such as NF-κB, resulting in decreased production of cytokines and therefore decreased expression of cell adhesion molecules and an attenuated inflammatory response (Navab *et al.*, 1991; Marui *et al.*, 1993; Berliner *et al.*, 1995). The significant inverse relation between lutein, lycopene and sICAM-1 are in line with the results of Martin *et al.* (2000). In this *in vitro* experiment, pre-treatment of human aortic endothelial cells (HAEC) with lycopene, lutein and β-carotene significantly reduced the expression of sICAM-1. However, in the present study we did not observe significant inverse associations between β-carotene, α-tocopherol and sICAM-1 as reported in *in vitro* studies (Martin *et al.*, 2000; Wu *et al.*, 1999). Lycopene and lutein have recently received attention for their potential role in preventing cardiovascular disease in humans. A low serum and adipose tissue lycopene concentration are associated with an excess incidence of acute coronary events, stroke and myocardial infarction, respectively (Rissanen *et al.*, 2001; Kohlmeier *et al.*, 1997). Furthermore, both plasma lycopene and lutein are associated with early atherosclerosis, as measured by intima-media thickness (Rissanen *et al.*, 2000; Dwyer *et al.*, 2001). Our regression analyses shows that serum lutein and lycopene concentrations at the 10^{th} percentile are associated with approximately 7% higher sICAM-1 concentrations than serum concentrations at the 90^{th} percentile. The relevance of a difference of approximately 16–18 ng/mL in sICAM-1 concentrations between the 10^{th} and 90^{th} percentiles of serum carotenoids is difficult to estimate. In a study of Blankenberg *et al.* (2001) patients with coronary heart disease with future death from cardiovascular disease had 66 ng/mL higher concentrations of sICAM-1 in comparison with patients with coronary heart disease who stayed alive during the study period. In a study of Ridker *et al.* (1998)

healthy subjects at baseline in the highest quartile of sICAM-1 (> 260 ng/mL) had 80% higher risk of myocardial infarction than subjects in the lowest quartile (< 193 ng/mL). Malik *et al.* (2001) concluded that measurement of adhesion molecules is unlikely to add much predictive information to that provided by more established risk factors on coronary heart disease. However, these data should not reduce the enthusiasm for targeting endothelium bound sICAM-1 as a potential method for therapy of cardiovascular disease as suggested by others (Ridker, 2001).

Our results did not confirm a protective effect of antioxidants on endothelial function measured by FMD measurement. Only zeaxanthin showed an inverse association, probably a chance finding. On the other hand, we found significant inverse associations between CRP, sICAM-1 and FMD.

In this study, the acute phase reactant C-reactive protein (CRP) was measured as marker of systemic inflammation. Surprisingly, because a negative association was expected, we observed a positive association between α-tocopherol and CRP in men. De Maat *et al.* (2000) found also a positive correlation between α-tocopherol concentrations and interleukin-6 (Il-6), another inflammation marker. At the moment these observations cannot be explained.

Previous studies found strong associations between β-carotene and CRP (Erlinger *et al.*, 2001; Kritchevsky *et al.*, 2000). We could confirm this positive association in the total group and we reported an inverse relation between lycopene and CRP in men only. Comparable with the results of Erlinger *et al.* (2001) we found an inverse relation between β-carotene and leucocytes. It has been suggested that in studying the relation between fruit and vegetable intake and cardiovascular disease risk, inflammation processes have to be taken into account as an confounding factor (Erlinger *et al.*, 2001; Kritchevsky, 1999; Kritchevsky *et al.*, 2000). The inverse relations between carotenoids and inflammation markers can explained by the fact that concentrations of carotenoids could be a reflection of inflammatory processes or possible anti-inflammatory properties of carotenoids.

In summary, we found inverse associations between lutein, lycopene and sICAM-1. β-Carotene was inverse related to leucocytes and CRP. Vitamin C was inverse related to CRP and α-tocopherol was positively associated with CRP. These inverse relations between carotenoids, vitamin C and sICAM-1, CRP and leucocytes may help to explain the possibly protective effect of carotenoids and

vitamin C on atherosclerosis through an influence on inflammatory processes and endothelial function.

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Chapter 6

Substantial decreased carotenoid concentrations, as caused by sucrose polyesters, have no impact on chronic disease markers

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Submitted

Abstract

Background: Excessive consumption of calories and fat increases the risk for obesity. Replacement of fatty foods by foods containing low- or zero-energy fat substitutes may help in reducing the energy intake of the population. Snacks containing sucrose polyesters (SPE) as dietary fat replacer are now on the market in the US. SPE products have been shown to lower concentrations of serum carotenoids in short term studies. Experimental studies on the longer-term effects on health of decreased carotenoid concentrations are lacking.

Methods: We performed a 1-year randomized, double blind, placebo-controlled parallel trial. We randomly assigned 380 subjects to the treatment with intakes of 0, 7, 10 or 17 g/d SPE. SPE was given in the form of spreads (daily consumption), chips (snackwise consumption), or both. SPE products were enriched with vitamin A, D, E and K according to the FDA regulation for olestra. We compared the groups with regard to body weight, serum carotenoids, vitamins and markers of oxidative damage (comet assay, LDL oxidation, carbonyl proteins, 8-epi-prostaglanins $F_{2\alpha}$, minimal erythema dose), eye health (macula pigment density), cardiovascular health (serum lipids, flow-mediated vasodilation) and immune status (antibodies against hepatitis B vaccination).

Results: After 1 year of intervention, serum lipid adjusted carotenoids showed the largest decrease in the group consuming both SPE chips and SPE spread (17 g/d) compared with the control group (α-carotene 33%; β-carotene 31%, lycopene 24%, β-cryptoxanthin 18%, lutein 18% (all $P < 0.001$) and zeaxanthin 13% ($P < 0.05$)). Consumption of SPE spread (10 g/d SPE) or SPE chips (7 g/d SPE) decreased carotenoid concentrations by 11% to 29% and 0.2% to 21%, respectively. In comparison with the control group serum lipid adjusted α-tocopherol decreased significantly by 6–8% (all $P < 0.001$) in all SPE groups. No negative effects were observed on markers of oxidation, eye health, cardiovascular health, and immune status despite the decreased concentrations of carotenoids.

Conclusion: This study suggests that substantial decreases in serum carotenoid concentrations do not imply an increased risk for chronic diseases.

Introduction

A high intake of fat is associated with an increased risk of obesity. Replacement of fatty foods by foods containing low- or zero-energy fat substitutes may be effective in reducing the energy intake of the population. A few years ago, low fat savory snacks containing sucrose polyester (SPE) as fat replacer (Olean®, Olestra) were introduced on the US market. It has been shown that products with SPE fat substitutes can lower concentrations of serum carotenoids (Weststrate and Van het Hof, 1995) and fat-soluble vitamins (Kelly *et al.*, 1998; Schlagheck *et al.*, 1997). Since SPEs hampers the absorption of fat soluble nutrients, the FDA requires the addition of vitamins A, D, E, and K to foods made with olestra (Federal Register, 1996). Addition of carotenoids was not mandatory because health effects of carotenoids are less well understood (Rock, 1997).

Carotenoids are fat-soluble compounds and occur naturally in fruit and vegetables. In addition to the best established function of β-carotene and to a lesser extent α-carotene and β-cryptoxanthin as precursor of vitamin A (Van Vliet *et al.*, 1996; Parker, 1996), carotenoids have other biological functions and actions (Van den Berg *et al.*, 2000). Carotenoids demonstrate *in vitro* antioxidant activity (Palozza and Krinsky, 1992; Sies and Krinsky, 1995; Krinsky, 1998), protect the skin through their singlet oxygen-quenching capacity (Mathews-Roth, 1993). Furthermore, cell-to-cell communication (Stahl and Sies, 1998) and immunomodulatory effects (Fuller *et al.*, 1992; Santos *et al.*, 1996) have been reported. Accumulation of lutein and zeaxanthin in the macula pigment of the retina could protect the eye by blue light filtering and singlet oxygen quenching (Seddon *et al.*, 1994). It has been suggested that carotenoids through these biological functions and actions may protect against degenerative diseases, for example cardiovascular disease (Cooper *et al.*, 1999; Kohlmeier and Hastings, 1995), cancer (Cooper *et al.*, 1999; Van Poppel and Goldbohm, 1995), and age-related macular degeneration (Cooper *et al.*, 1999; Seddon *et al.*, 1994).

In addition to SPE products, more foods which adversely affect carotenoids may be introduced. For instance, cholesterol lowering phytosterol and stanol products also reduce carotenoid concentrations to a lesser extent (Weststate and Meijer, 1998; Plat and Mensink, 2001).

The longer-term effects as well as the functional consequences of decreased carotenoid concentrations have not been previously studied in detail in human

intervention studies. Therefore, we conducted a long-term human intervention trial to investigate the possible effects of SPE consumption on serum carotenoid and fat soluble vitamin concentrations and markers of oxidative damage and also functional markers of eye, cardiovascular health and immune status.

Methods

Subjects and study design

Between June 29, 1998 and September 8, 1999 we conducted a double blind, randomized, placebo-controlled, parallel design trial with 4 treatment groups to study the effects of long-term SPE consumption. The study was performed according to ICH guidelines for good clinical practice. The protocol was approved by an external Medical Ethical Committee.

Subjects were recruited through advertizing. Respondents who expressed potential interest received information and a questionnaire on fruit and vegetable consumption and lifestyle (Broekmans *et al.*, in press). The main exclusion criteria were age not between 18-75 years, pregnancy (wish) and/or lactation, serum cholesterol > 7.5 and/or triacylglycerol > 2.3 mmol/L if not under stabilized hypercholesterolemia/hyperlipidemia treatment, anticoagulant therapy, and vegetarians and vegans.

A total of 2734 out of 6900 questionnaires were returned. Respondents ranked in the highest quintile of fruit and vegetable consumption (n = 547) and those in the lowest tertile (n = 775) were invited for an oral briefing. This pre-selection was made in order to select volunteers with a relatively high and low serum carotenoid status. Five hundred and eighty-nine volunteers gave their informed consent and filled in a questionnaire on personal data, life style, medical history and dietary habits. Fifty-four volunteers were excluded on criteria for serum cholesterol and/or triacylglycerol. One hundred and fifty-five volunteers withdrew before the start of the study, leaving a total of 380 volunteers in this study at day 01.

Subjects were randomly assigned to one of the four treatment groups. High/low fruit and vegetable consumption, age, gender and smoking habit (yes/no) were used as randomization parameters. Eighty-five subjects entered the study in June 1998, 115 subjects entered in August 1998 and 180 subjects entered the trial in September 1998.

The subjects consumed SPE in different concentrations and food products, e.g. spread and chips (deep-fried potato slices). The four treatment groups consumed control spread and control chips (control group), or SPE spread and control chips (SPE spread group), or control spread and SPE chips (SPE chips group), or SPE spread and SPE chips (SPE spread + SPE chips group), respectively. The content of SPE and triacylglycerol (TAG) of the products is given in Table 1. All data in Table 1 are weighed averages over all portions consumed from the various product batches.

Table 1 Composition of study substances in units per gram study substance (averages weighed over the number of portions consumed per batch)

	Control spread	SPE spread	Control chips	SPE chips
Sucrose Polyester (g)	–	0.501	–	0.244
Triacylglycerol (g)	0.330	0.344	0.327	0.004
Vitamin A (μg)[1]	10.5	47.2	–	36.0
Vitamin D (ng)[2]	69.0	145	–	129
Vitamin E (mg)[3]	0.17	1.17	0.06	0.86
Vitamin K1 (μg)[4]	–	3.4	–	3.0
Carotene (μg)	6	6	4	4
Total tocopherols (μg)	528	183	433	221

[1] 94.2 μg retinyl palmitate/g SPE in SPE spread and 147.5 μg retinyl palmitate/g SPE in SPE chips (FDA requirement: 93.0 μg retinyl palmitate/g SPE). [2] 290 ng cholecalciferol/g SPE in SPE spread and 529 cholecalciferol ng/g SPE in SPE chips (FDA requirement: 300 ng cholecalciferol /g SPE). [3]2.34 mg d-α-tocopheryl acetate/g SPE in SPE spread and 3.51 mg d-α-tocopheryl acetate/g SPE in SPE chips (FDA requirement: 2.07 mg d-α-tocopheryl acetate/g SPE). [4]6.7 μg 3-phytyl menadione/g SPE in SPE spread and 12.2 μg 3-phytyl menadione/g SPE in SPE chips (FDA requirement: 8.0 μg 3-phytyl menadione/g SPE).

Chips containing SPE (olestra) were bought on the US market. Control chips were bought on the Dutch and US markets. All chips were repacked in neutral bags in weekly portions of 200 gram. Spreads were produced on pilot plant votator equipment at Unilever R&D Vlaardingen, according to regular spread processing procedures. The test spread contained 50% SPE and 35% TAG. The control spread was a low-fat spread containing 35% TAG. More detailed data are given in Table 1. The average portion size of one tub of each spread was 140 gram, intended for

consumption within one week. SPE raw materials for use in the test spreads were also produced at Unilever according to methods comparable to those described for the production of Olestra.
The spreads were consumed as part of a normal dietary intake with an intended daily dosage of 20 g/d (thus either 0 or 10 g SPE per day, plus 7 g TAG). Subjects were instructed not to use the spread for cooking or baking. The chips were to be consumed as a snack in an amount of 200 g/wk, without a daily usage requirement. This lead to an average consumption of 7 g/d SPE in test chips or 9 g/d TAG in control chips. The SPE dose of the chips is comparable to the dose used by the FDA to estimate chronic (lifetime) Olestra intake by the 90th percentile snack eater. We have estimated the SPE intake from spreads at an intake level, somewhat above the fat intake from an average margarine intake in the US, which is 11 g/d of margarine, or about 8 g/d of fat.
The SPE spreads were enriched with vitamin A, D, E and K at vitamin/SPE levels required by the FDA for the use of olestra in chips. It was assumed that the same vitamin levels were present in the SPE chips taken from the market, but analyses showed that the test chips on average in fact contained 170% of the mandatory vitamin levels. The control spreads were enriched with vitamins A and D at levels (on product) as required by the Dutch law. The control chips were not fortified. The detailed vitamin composition of the study substances is given in Table 1.
Distribution of the study products occurred in monthly intervals with four 140 g tubs (weekly portion) of spread and four bags of 200 g chips. Returned spread tubs and chips bags were counted and registered and when a tub or a bag was not empty, the amount left was weighed and registered.
Fasting morning blood samples were taken at baseline and after 13, 26 and 52 weeks. Serum and plasma was stored at –20°C or –80°C.
During the 52 week study, 39 subjects dropped out because of several reasons, generally not treatment related: control group 10; SPE chips group 8; SPE spread group 9; SPE spread + SPE chips group 12.

Analyses of products
The total fat content and the content of SPE and TAG in the products were analyzed using gel permeation chromatography after extraction of the total fat phase, according to internal methods. The vitamin content of the study substances

was analyzed at the RIKILT-DLO institute in Wageningen, The Netherlands. Following extraction with heptane the carotene and total tocopherols content were analyzed by HPLC. Several other analyses were carried out to check the quality and consistency of the various product batches.
The SPE raw materials used for the production of test spreads were analyzed for all parameters used in the specification of olestra by the FDA. The stiffness and the degree of esterification (of fatty acids on sucrose) were slightly different from the specification, all other parameters were within the specifications. The composition of the olestra material in the test chips was not analyzed.

Body weight
Body weight was assessed by weighing subjects wearing indoor clothing, without shoes, wallet and keys at monthly intervals.

Biochemical analyses
Serum carotenoids (CVs 3.7–14.9%), retinol (2.5%), α-tocopherol (2.8%) and vitamin C (10%) were quantified by reversed phase HPLC (Broekmans *et al.*, in press). Vitamin D (25-hydroxycholecalciferol) was quantified by competitive protein-binding assay according to Edelstein *et al.* (1974) with slight modifications (CV 11%). Vitamin K1 (Phylloquinone) in serum was assessed by HPLC with 2',3'-dihydrophylloquinone as internal standard (CV 8%).
LDL-oxidation was measured according to Esterbauer *et al.* (1989). DNA damage, measured as the comet assay after induction with hydrogen peroxide (H_2O_2), was conducted as described by Collins *et al.* (1993). Plasma 8-epi-prostaglandin $F_{2\alpha}$ was measured according to a method described by Nourooz-Zadeh *et al.* (1995) with some modifications. Plasma protein carbonyl content was quantified using an enzyme-linked immunosorbent assay as previously described (Buss *et al.*, 1997).
Subjects known not to be vaccinated against hepatitis B received i.m. (m. Deltoideus) standard doses (20 μg HbsAg/dose) of hepatitis B vaccine (Engerix-B®, SmithKline Beecham Farma BV, Rijswijk, The Netherlands) on week 39, 43, and 48 of the study. Anti-HBs antibodies were determined using ELISA in serum samples at week 39, 43, 48 and 52 (Hepanostika® Anti-HBs microELISA test kit, Organon Teknika N.V. Turnhout, Belgium).

Total cholesterol, HDL cholesterol and triacylglycerol were analyzed by enzymatic techniques (Boehringer, Mannheim, Germany). LDL cholesterol was calculated according to the Friedewald formula (Friedewald *et al.*, 1972).

Other measurements

Macular pigment density was measured as a marker of lutein and zeaxanthin in the eye by spectral reflectance analysis with the Utrecht Retinal Densitometer (Van de Kraats *et al.*, 1996). A previous study using the same method showed a within subject variation coefficient of 17% (Berendschot *et al.*, 2000).

The minimal erythema dose (MED) quantifies the sensitivity of the skin to solar ultraviolet (UV) irradiation by exposing the skin to increasing UV dosages as described previously (Broekmans *et al.*, submitted) with a CV < 12.5%.

Flow-mediated vasodilation (FMD) of the brachial artery was measured as a marker of endothelial function (De Roos *et al.*, 2001). The diameter of the artery at rest and at maximum vasodilation was used to calculate the percentage FMD.

Adverse events were classified according to "The ICD-10: International statistical classification of diseases and related health problems 10th revision" WHO 1993. Gastrointestinal symptoms were reported by a self-reporting questionnaire.

Statistical analyses

Data were expressed as mean ± standard deviation (SD) for each treatment. In order to correct for changes in serum lipid concentrations, we present percentual changes in lipid standardized carotenoid and α-tocopherol concentrations. Serum lipid adjusted carotenoid concentrations were calculated as follows: [carotenoid concentration / (total cholesterol + triacylglycerol)]. Overall, differences in changes (week 52 – week 0) between groups were tested by ANOVA with treatment as factor using the F-test on a $\alpha = 5\%$ significance level (PROC GLM, SAS/STAT software, version 6.12, SAS Institute, Cary, N.C.). If a significant overall treatment effect was detected, the least significant difference (LSD) using a two-sided significance level of $\alpha = 5\%$, based on the ANOVA mean squared error, was used to determine which groups were different. If data were not normally distributed, data were natural log transformed before ANOVA.

Antibodies against Hepatitis B are presented at week 52. Effects were evaluated by assessment of the amount of antibodies formed and by counting the number of

subjects who were seroconverted (Hbs antibodies > 10 IU/L). If volunteers were already seroconverted for hepatitis B at week 39 they were excluded from statistical analysis.

Results

Baseline characteristics

The baseline characteristics of the four treatment groups were similar (Table 2). Three hundred and forty one subjects (164 men and 177 women) completed the study.

Table 2 Baseline characteristics of the 341 volunteers, who completed the study[1]

	Control	SPE chips	SPE spread	SPE spread + SPE chips
Men/women (*n*/*n*)	43/43	43/44	38/49	40/41
BMI (kg/m^2)	24.4 ± 4.2	25.0 ± 4.5	24.8 ± 4.0	24.2 ± 3.4
Age (y)	42.6 ± 14.5	41.6 ± 14.0	43.0 ± 13.4	40.6 ± 14.0
Low F&V consumption (%)[2]	57	56	55	60
Smoking (%)	33	29	30	27
SPE intake (g/day)	0	7	10	17

[1] Mean ± standard deviation unless stated otherwise. [2] Lowest tertile of ranked respondents of fruit and vegetable consumption questionnaire (see method section).

Adverse events and gastrointestinal effects

No differences in adverse events were observed between the treatment groups. Three serious adverse events occurred, that were considered to be unlikely related to the treatment (control group 1; SPE spread group 1; SPE spread + SPE chips group 1). The GI questionnaire showed no clear effects of SPE consumption (details are available on request).

Compliance and eating moment

Consumption of spread and chips was evaluated after 13, 26 and 52 weeks and did not differ between the groups. The overall compliance was good, > 98.8% in all groups. Spreads were consumed mostly on bread. Approximately 10% of the

consumers also used the spread with dinner. Chips were mostly consumed as a snack in a frequency of three to five times per week.

Effects on body weight

During the year the mean body weight (± SD) increased with 1.5 ± 2.9 kg (2.0 ± 3.8%) in the control group, 0.5 ± 3.4 kg (0.7 ± 4.5%) in the SPE chips group, 1.2 ± 2.4 kg (1.6 ± 3.2%) in the SPE spread group and 0.5 ± 3.2 kg (0.7 ± 4.2%) in the SPE spread + SPE chips group ($P > 0.05$).

Effects on carotenoids and vitamins

Figure 1 shows that mean carotenoid concentrations were significantly decreased after 52 weeks and for most of the carotenoids a steady state was achieved at week 13. Baseline concentrations of carotenoids and the time required to reach a steady state were comparable between the groups consuming SPE.

Figure 1 Mean (± SE) carotenoid concentrations at week 0, 13 and 52 in the SPE spread + SPE chips group ($n = 81$)

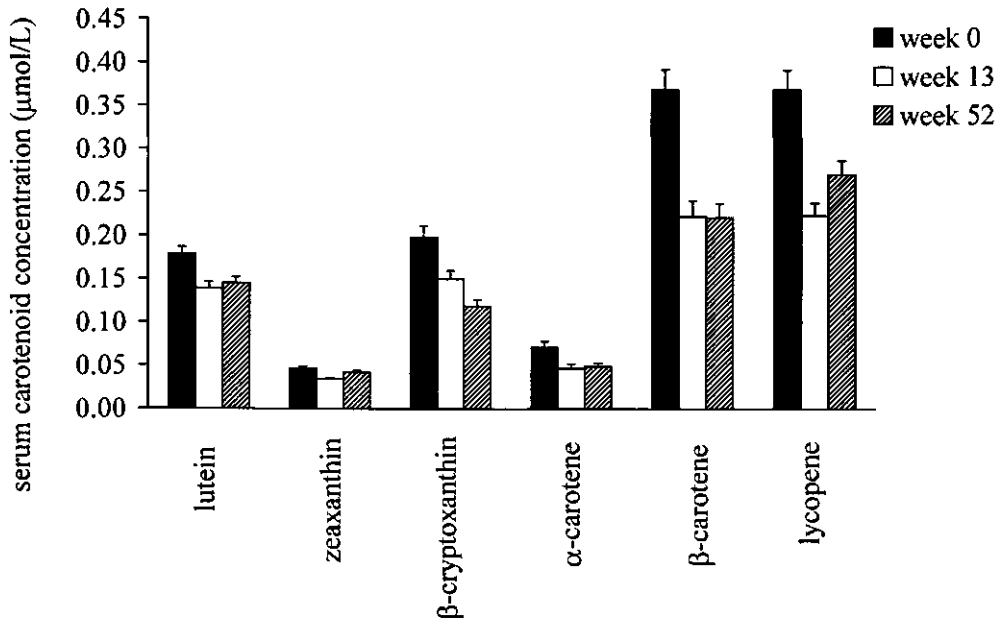

Table 3 presents the mean lipid standardized values of carotenoids and α-tocopherol for the treatment groups.

Table 3 Effects on serum carotenoid (nmol/mmol) and α-tocopherol concentrations (μmol/mmol) corrected for total cholesterol (TC) and triacylglycerol (TG) following one year consumption of control products, SPE chips, SPE spread and both SPE spread and chips[§]

	Time	Control	SPE chips	SPE spread	SPE spread + SPE chips
Lutein/	Week 0	25.5 ± 9.4	27.1 ± 12.1	26.4 ± 10.3	28.2 ± 11.3
(TC+TG)	Week 52	24.7 ± 8.5	24.0 ± 10.2	23.0 ± 10.0	22.3 ± 10.1
	Change	−0.8 ± 6.7[3,4]	−3.0 ± 7.0[4]	−3.4 ± 7.0[1,4]	−5.9 ± 9.0[1,2,3]
Zeaxanthin/	Week 0	7.2 ± 3.3	7.8 ± 3.0	7.7 ± 3.4	7.4 ± 3.0
(TC+TG)	Week 52	7.2 ± 3.3	7.0 ± 2.8	6.7 ± 2.4	6.5 ± 3.0
	Change	0.02 ± 2.4[2,3,4]	−0.8 ± 2.3[1]	−0.9 ± 2.6[1]	−0.9 ± 2.7[1]
β-Crypto-	Week 0	28.9 ± 20.3	30.0 ± 16.8	29.8 ± 17.7	31.1 ± 18.7
xanthin/	Week 52	23.6 ± 17.6	25.2 ± 18.4	19.4 ± 11.2	18.0 ± 10.0
(TC+TG)	Change	−5.3 ± 10.0[3,4]	−5.2 ± 12.9[3,4]	−10.4 ± 12.3[1,2]	−13.0 ± 15.0[1,2]
α-Carotene/	Week 0	9.8 ± 8.4	10.8 ± 9.7	10.7 ± 8.5	11.2 ± 10.3
(TC+TG)	Week 52	9.5 ± 5.4	8.5 ± 6.4	7.7 ± 7.6	7.7 ± 6.4
	Change	−0.3 ± 6.6[2,3,4]	−2.3 ± 5.7[1]	−3.1 ± 7.0[1]	−3.5 ± 7.6[1]
β-Carotene/	Week 0	55.5 ± 32.8	53.5 ± 30.8	59.5 ± 40.6	57.6 ± 35.1
(TC+TG)	Week 52	48.9 ± 26.3	42.7 ± 27.9	38.0 ± 28.7	34.2 ± 24.3
	Change	−6.6 ± 20.4[2,3,4]	−11.6 ± 17.5[1,3,4]	−21.5 ± 29.7[1,2]	−23.4 ± 26.1[1,2]
Lycopene/	Week 0	54.5 ± 26.7	52.3 ± 24.6	55.7 ± 29.2	57.1 ± 32.4
(TC+TG)	Week 52	50.6 ± 24.9	44.0 ± 20.6	43.2 ± 26.7	41.1 ± 22.7
	Change	−3.9 ± 21.6[3,4]	−8.4 ± 21.7[4]	−12.5 ± 23.6[1]	−16.1 ± 23.6[1,2]
α-Toco-	Week 0	3.9 ± 0.6	4.2 ± 0.6	4.1 ± 0.7	4.0 ± 0.5
pherol/	Week 52	3.8 ± 0.5	3.8 ± 0.4	3.8 ± 0.6	3.7 ± 0.5
(TC+TG)	Change	−0.05 ± 0.4[2,3,4]	−0.3 ± 0.4[1]	−0.3 ± 0.4[1]	−0.4 ± 0.4[1]

[§]Mean ± standard deviation. [1] Significantly different from control; [2] Significantly different from SPE chips; [3] Significantly different from SPE spread; [4] Significantly different from SPE spread + SPE chips.

The greatest reductions were observed for α-carotene (–0.02 ± 0.05 μmol/L), β-carotene (–0.15 ± 0.17 μmol/L) and lycopene (–0.10 ± 0.15 μmol/L) in the SPE spread + SPE chips group. Compared with the control group lipid standardized concentrations of carotenoids decreased by 33% ($P < 0.0001$) for α-carotene, followed by β-carotene (31%; $P < 0.0001$), lycopene (24%; $P < 0.001$), β-cryptoxanthin (18%; $P < 0.0001$), lutein (18%; $P < 0.0001$) and zeaxanthin (13%; $P < 0.001$) in the SPE chips + SPE spread group.

In the SPE spread group α-carotene, β-carotene and lycopene decreased by 0.02 ± 0.05, 0.13 ± 0.19 and 0.07 ± 0.17 μmol/L, respectively. Lipid standardized serum carotenoid concentrations decreased significantly by 11% for lutein and zeaxanthin ($P < 0.05$), 14% ($P < 0.05$) for β-cryptoxanthin, 19% ($P < 0.05$) for lycopene, 25% ($P < 0.0001$) for β-carotene and 29% ($P < 0.0001$) for α-carotene ($P < 0.0001$) in comparison with the control group.

Lipid standardized serum zeaxanthin, α-carotene, β-carotene decreased significantly by 11% ($P < 0.05$), 21% ($P < 0.05$), 12% ($P < 0.05$) in the SPE chips group in comparison with the control group, respectively. No significant differences between the control group and SPE chips group were found for lutein, β-cryptoxanthin and lycopene.

Despite supplementation of test products with vitamin E, lipid standardized serum α-tocopherol decreased by 8% ($P < 0.0001$) in the SPE spread + SPE chips group, 6% ($P < 0.001$) in the SPE spread group and 7% ($P < 0.0001$) in the SPE chips group compared to the control group.

The changes in vitamin A (retinol), C, D and K concentrations were not significantly different between the groups (Table 4).

Table 4 Effects on serum vitamin A (retinol), D and K and blood vitamin C concentrations following one year consumption of control products, SPE chips, SPE spread and both SPE spread and chips[§]

	Time	Control	SPE chips	SPE spread	SPE spread + SPE chips
Retinol	Week 0	2.01 ± 0.42	2.07 ± 0.39	2.03 ± 0.52	2.07 ± 0.49
(μmol/L)	Week 52	2.04 ± 0.42	2.19 ± 0.37	2.07 ± 0.50	2.11 ± 0.49
	Change	0.03 ± 0.27	0.12 ± 0.32	0.04 ± 0.31	0.05 ± 0.31
25-OH-	Week 0	77 ± 24	81 ± 29	77 ± 22	78 ± 27
Vitamin D	Week 52	87 ± 24	93 ± 28	91 ± 30	91 ± 25
(nmol/L)	Change	9 ± 15	12 ± 18	15 ± 22	13 ± 18
Vitamin C	Week 0	43.9 ± 13.6	45.2 ± 14.9	46.2 ± 14.5	45.3 ± 11.3
(μmol/L)	Week 52	52.4 ± 15.6	54.6 ± 15.9	58.0 ± 15.7	54.9 ± 13.6
	Change	8.6 ± 12.8	9.4 ± 13.3	11.8 ± 12.9	9.6 ± 14.1
Vitamin K1	Week 0	1378 ± 1998	1212 ± 1060	1410 ± 2278	1325 ± 2399
(pmol/L)	Week 52	1100 ± 966	1149 ± 951	967 ± 825	933 ± 593
	Change	−228 ± 2053	−52 ± 1412	−454 ± 1979	−392 ± 2340

[§]Mean ± standard deviation.

At three time points post study (15-35; 41-58; 64-91 days after study) mean serum carotenoids and α-tocopherol concentrations were not significantly different between the control group and SPE spread + SPE chips group.

Effects on serum lipids

Total cholesterol in the group consuming SPE chips increased significantly more (8.5%) than in the control (2.6%; $P < 0.05$) and SPE spread + SPE chips groups (1.6%; $P < 0.001$).

Table 5 Effects on serum lipid concentrations following one year consumption of control products, SPE chips, SPE spread and both SPE spread and chips[§]

	Time	Control	SPE chips	SPE spread	SPE spread + SPE chips
Total	Week 0	5.61 ± 1.00	5.47 ± 0.93	5.64 ± 0.90	5.43 ± 0.98
cholesterol	Week 52	5.71 ± 1.02	5.91 ± 1.06	5.85 ± 0.88	5.47 ± 0.84
(mmol/L)	Change	0.10 ± 0.62[2]	0.42 ± 0.74[1,4]	0.22 ± 0.62	0.04 ± 0.61[2]
LDL	Week 0	3.85 ± 0.98	3.78 ± 0.96	3.88 ± 0.93	3.67 ± 0.91
(mmol/L)	Week 52	3.89 ± 0.97	4.05 ± 1.08	4.06 ± 0.94	3.64 ± 0.87
	Change	0.04 ± 0.57[2]	0.27 ± 0.73[1,4]	0.17 ± 0.63[4]	−0.03 ±0.65[2,3]
HDL	Week 0	1.61 ± 0.41	1.54 ± 0.41	1.60 ± 0.42	1.60 ± 0.47
(mmol/L)	Week 52	1.64 ± 0.43	1.61 ± 0.44	1.64 ± 0.43	1.62 ± 0.39
	Change	0.03 ± 0.25	0.06 ± 0.20	0.04 ± 0.23	0.02 ± 0.27
Triacylgly-	Week 0	1.125 ± 0.568	1.122 ± 0.466	1.141 ± 0.493	1.139 ± 0.692
cerol	Week 52	1.220 ± 0.580	1.401 ± 0.684	1.182 ± 0.509	1.227 ± 0.786
(mmol/L)	Change	0.095 ± 0.525	0.283 ± 0.497[3,4]	0.041 ± 0.417[2]	0.088 ± 0.467[2]

[§]Mean ± standard deviation. [1] Significantly different from control; [2] Significantly different from SPE chips; [3] Significantly different from SPE spread; [4] Significantly different from SPE spread + SPE chips.

Changes in LDL cholesterol coincided with those in total cholesterol: LDL cholesterol increased significantly more in the SPE chips group (8.4%) compared to the control group (3.2%; $P < 0.05$) and the SPE spread + SPE chips group (0.8%; $P < 0.05$). Also LDL cholesterol increased significantly more in the SPE spread group (6.4%; $P < 0.05$) in comparison with SPE spread + SPE chips group. The changes in HDL cholesterol were not significantly different between the four groups at week 52 (Table 5).

Increases of triacylglycerol (TAG) were significantly higher in the group consuming SPE chips (31.4%) compared to the SPE spread group (9.6%; $P < 0.05$) and SPE spread + SPE chips group (13.8%; $P < 0.05$). Consumers of SPE spread + SPE chips or only SPE spread did not have a significantly different change after 52 weeks of triacylglycerol, total, and LDL cholesterol concentrations compared to the control group (Table 5).

Effects on oxidation markers

No differences were found between the four treatment groups for lagtime (LDL-oxidation), protein carbonyls (protein oxidation), lipid peroxidation products (8-epi-prostaglanins $F_{2\alpha}$) and DNA damage as measured by the comet assay (Table 6). The mean changes from baseline in minimal erythema dose (MED) were not significantly different between the four groups. In comparison with the control group, the mean MED scores decreased by 8% and 6.3% in the SPE chips group and SPE spread + SPE chips group, respectively. The mean MED increased by 3% in the SPE spread group in comparison with the control group (Table 6).

Table 6 Effects on markers of oxidative damage (LDL-oxidation, protein carbonyls, 8-epi-prostaglanins $F_{2\alpha}$, comet assay and MED) following one year consumption of control products, SPE chips, SPE spread and both SPE spread and chips[§]

	Time	Control	SPE chips	SPE spread	SPE spread + SPE chips
Lagtime	Week 0	90.7 ± 7.2	89.9 ± 6.1	90.2 ± 7.8	90.1 ± 6.3
(minutes)	Week 52	88.8 ± 6.6	89.0 ± 6.5	88.6 ± 7.6	88.8 ± 7.2
	Change	−1.6 ± 5.8	−0.9 ± 4.9	−1.5 ± 5.1	−1.3 ± 4.7
Protein	Week 0	0.36 ± 0.04	0.36 ± 0.04	0.36 ± 0.04	0.35 ± 0.04
carbonyls	Week 52	0.35 ± 0.04	0.36 ± 0.03	0.36 ± 0.04	0.36 ± 0.04
(nmol/mg)	Change	−0.01 ± 0.03	0 ± 0.04	0 ± 0.03	0 ± 0.03
8-epi-prosta-	Week 0	22.6 ± 7.6	22.4 ± 7.9	25.3 ± 11.6	23.8 ± 9.9
glandins $F_{2\alpha}$	Week 52	21.4 ± 8.5	21.2 ± 7.6	21.4 ± 7.5	23.8 ± 10.9
(ng/L)	Change	−1.3 ± 7.9	−1.2 ± 8.5	−3.9 ± 10.9	0.1 ± 10.7
Comet assay	Week 0	13.5 ± 11.2	14.1 ± 11.7	12.1 ± 13.8	13.7 ± 12.9
(H_2O_2)	Week 52	6.1 ± 7.0	6.8 ± 8.2	6.8 ± 7.1	6.6 ± 9.0
	Change	−7.5 ± 12.6	−7.3 ± 14.9	−5.3 ± 15.3	−7.1 ± 15.2
MED (× sMED)	Week 0	0.56 ± 0.20	0.56 ± 0.21	0.56 ± 0.19	0.56 ± 0.19
	Week 52	0.60 ± 0.26	0.55 ± 0.23	0.61 ± 0.24	0.56 ± 0.23
	Change	0.04 ± 0.18	−0.01 ± 0.17	0.05 ± 0.18	0.00 ± 0.16

[§]Mean ± standard deviation.

Effects on functional markers

The changes in macula pigment (MP) density were not significantly different between the groups. The median values (25^{th}-75^{th} percentiles) of the individual percentual changes of macular pigment density were 6.5% (−8.1 – 28.8), 3.8% (−14.3 – 34.7), 5.1% (−9.9 – 34.5) and 1.7 % (−16.9 – 36.0) in the control, SPE chips, SPE spread and SPE spread + SPE chips group, respectively (Table 7). Because of technical problems with the densitometer, 83 measurements were not performed in week 52. But within a 10-week period after week 52, MP density measurement was repeated in 45 subjects. Exclusion of these data did not affect previous results.

There were no significant differences in changes in mean calculated endothelial function between the treatment groups as measured by flow-mediated vasodilation (Table 7). SPE consumption had no effects on immune function. The number of seroconverted subjects was not significantly different between the groups, nor was the antibody titer against Hepatitis B (Table 7).

Table 7 Effects on markers of eye, cardiovascular health and immune status following one year consumption of control products, SPE chips, SPE spread and both SPE spread and chips[§]

	Time	Control	SPE chips	SPE spread	SPE spread + SPE chips
MP density	Week 0	0.32 ± 0.14	0.33 ± 0.14	0.34 ± 0.15	0.31 ± 0.16
	Week 52	0.36 ± 0.15	0.37 ± 0.16	0.38 ± 0.14	0.34 ± 0.17
	Change	0.03 ± 0.10	0.04 ± 0.15	0.03 ± 0.12	0.02 ± 0.13
FMD (%)	Week 0	4.72 ± 3.37	4.49 ± 5.53	4.99 ± 5.60	4.56 ± 3.99
	Week 52	4.60 ± 3.70	3.62 ± 4.03	4.48 ± 4.33	4.05 ± 3.74
	Change	0.17 ± 5.04	−1.00 ± 6.18	0.13 ± 5.78	−0.64 ± 5.01
Titer Hepatitis B (IU/L)	Week 52	449.7 ± 984.9	504.3 ± 1033.8	656.7 ±1513.9	383.9 ± 745.6
Sero/non-Converted (*n*/*n*)[a]	Week 52	55/12	63/10	60/15	46/10

[§]Mean ± standard deviation unless stated otherwise [a] seroconverted (Hbs antibodies > 10 IU/L).

Discussion

This study provides, perhaps surprising, evidence that substantial serum carotenoid decreases do not imply increased risk for chronic diseases.

Among the strengths of our study are the large study group, double blind placebo-controlled 1-year study design, good reported compliance and the free-living conditions. Overall, a dose-response existed between SPE consumption and serum carotenoid concentrations. However, this difference in serum carotenoid changes could also be caused by consumption of different food products and consumption patterns. The reductions in serum carotenoids following SPE consumption reported here are comparable with the reductions reported for short-term trials (Weststrate and Van het Hof, 1995; Koonsvitsky *et al.*, 1997; Schlagheck *et al.*, 1997; Kelly *et al.*, 1998). The most lipophylic hydrocarbon carotenoid concentrations (α-carotene, β-carotene and lycopene) showed larger changes than the more polar oxygenated carotenoids lutein, zeaxanthin and β-cryptoxanthin. Results of short-term trials indicate that daily consumption of SPE may reduce the serum concentrations of carotenoids very rapidly, but a new steady state level is reached within four weeks. In this trial, we observed a steady state within 13 weeks, except for β-cryptoxanthin (Figure 1).

In the cohort of the Olestra Post Marketing Surveillance Study (OPMSS), reductions between baseline and follow-up of 1 year in the highest category of SPE consumption were 21% for β-carotene (compared with 7% for nonconsumers) and 7% for lycopene (compared with no change for nonconsumers). None of these effects in this analysis was significant and there were no dose-response relationships between olestra and serum carotenoids (Thornquist *et al.*, 2000). These smaller reductions in serum carotenoids are most likely due to a lower SPE consumption.

In the present study, concentrations of lipid soluble vitamins retinol, 25-OH vitamin D and vitamin K1 were not affected by the study substances containing SPE enriched with vitamin A, D and K, suggesting that the mandatory enrichment with these vitamins was sufficient to offset any SPE effects on lipid soluble vitamin absorption, although the actual enrichment of SPE chips was higher than required by the FDA. However, we report that lipid adjusted serum concentrations of α-tocopherol were affected by SPE consumption irrespective of product format despite the enrichment with α-tocopheryl acetate. This suggests that an adjustment

of the FDA regulation for α-tocopherol enrichment of Olestra containing savory snacks might be considered, particularly because the enrichment of SPE chips was 70% higher than required. The major objective of this study was to examine the effects of substantial carotenoid decreases on possible markers of oxidative damage and functional markers related to eye, cardiovascular health and immune status. We did not observe any negative effects on these markers after 1 year. It should be noted that for almost all markers the relation between these markers and the final disease risk has to be established.

Results of published supplementation studies with carotenoids are not completely consistent regarding the effect on biomarkers of oxidative damage. We did not observe an effect on these markers after significant decreases of carotenoid concentrations. Our results are in line with the observation that olestra consumption is not associated with reduced macular pigment optical density in a free-living population (Cooper *et al.*, 2000). In this study serum lutein and zeaxanthin concentrations were not affected either, possibly because of a lower intake of SPE in comparison with our study. Both studies were limited by the duration of 1 year, however intervention studies which aimed to increase serum carotenoids show both an increase of MP density (Berendschot *et al.*, 2000; Johnson *et al.*, 2000) and MED (Stahl *et al.*, 2001; Stahl *et al.*, 2000) within several weeks. Johnson *et al.* (2000) showed that consumption of spinach and corn with the daily diet resulted in an increase of 0.07 (approximately 18%) in MP density after 15 weeks. In the study of Stahl *et al.* (2001) erythema was 40% lower in subjects consuming tomato paste, with serum lycopene being 2-fold higher after 10 weeks. A posteriori power analysis indicated that we could have detected a difference of 0.055 in MP density and 0.075 in MED between treatments. Our study showed that 18% decrease in serum lipid adjusted lutein and 24% decrease in serum lipid adjusted lycopene during 1 year did not have negative effects on MP density and MED.

The present study does not demonstrate any negative effects of lower concentrations of serum carotenoid and α-tocopherol on FMD. However, it should be noted that the within subject variability of FMD is large, about 50% of the mean response (De Roos, 2001). With our number of subjects we could detect a difference of 3.3%-points in FMD with a probability of 0.05 and a power of 0.80.

There are indications that the antioxidants β-carotene and vitamin E could have immuno-enhancing effects (Bendich, 1989; Hughes, 2001; Meydani, 1999). Our

results indicate no negative effects of carotenoid and α-tocopherol decreases on the primary adaptive immunity as measured by antibody production to a model infection mimicked by hepatitis B vaccination. In addition, we did not observe negative effects on clinical chemical parameters, hematological parameters, coagulation/fibrinolysis and inflammation markers (data not shown).

Although this study was not specifically designed to evaluate the possible body weight and cholesterol lowering effect of SPE when as dietary fat replacers, we did not observe a clear impact on serum lipids concentrations and body weight after daily SPE consumption during a year. In an observational study, Patterson *et al.* (2000) reported that frequent consumers of olestra, those with 2 servings of chips per week for a year, experienced a decrease of 10% in total serum cholesterol and a reduction in dietary fat intake. In the study of Patterson *et al.* (2000) heavy users lost about a pound of weight, while weight remained stable among non-consumers. These authors suggested that olestra consumption is probably an indicator of a healthier lifestyle in general.

In this study, we evaluated the effects in an average Dutch population, including subjects from subpopulations probably more at risk for the effects of carotenoid decreases such as elderly, smokers and habitual low fruit and vegetable consumers. Our observations show that in these subpopulations functional markers were not affected by the consumption of SPE either (data not shown).

In observational studies it has consistently been shown that higher serum carotenoid concentrations are related to a decreased risk of chronic diseases. However, intervention studies which aimed to increase serum carotenoids by supplementation with β-carotene could not confirm this protective effect against chronic diseases. Our study suggests that a substantial and prolonged decrease in serum carotenoids does not imply an increased risk for chronic diseases and that SPE consumption during one year has no beneficial effects on serum lipids or body weight.

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Chapter 7

Fruits and vegetables increase plasma carotenoids and vitamins and decrease homocysteine in humans

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Abstract

Observational epidemiologic studies have shown that a high consumption of fruits and vegetables is associated with a decreased risk of chronic diseases. Little is known about the bioavailability of constituents from vegetables and fruits and the effect of these constituents on markers for disease risk. Currently, the recommendation is to increase intake of a mix of fruits and vegetables ('five a day'). We investigated the effect of this recommendation on plasma carotenoids, vitamins and homocysteine concentrations in a 4-wk dietary controlled, parallel intervention study. Male and female volunteers (n = 47) were allocated randomly to either a daily 500 g fruit and vegetable ('high') diet or a 100 g fruit and vegetable ('low') diet. Analyzed total carotenoid, vitamin C and folate concentrations of the daily high diet were 13.3 mg, 173 mg and 228.1 μg, respectively. The daily low diet contained 2.9 mg carotenoids, 65 mg vitamin C and 131.1 μg folate. Differences in final plasma levels between the high and low group were as follows: lutein, 46% [95% confidence interval (CI) 28 – 64]; β-cryptoxanthin, 128% (98 – 159); lycopene, 22% (8 – 37); α-carotene, 121% (94 – 149); β-carotene, 45% (28 – 62); and vitamin C, 64% (51 – 77) ($P < 0.05$). The high group had an 11% (–18 to –4) lower final plasma homocysteine and a 15% (0.8 – 30) higher plasma folate concentration compared with the low group ($P < 0.05$). This is the first trial to show that a mix of fruits and vegetables, with a moderate folate content, decreases plasma homocysteine concentrations in humans.

Introduction

Observational epidemiologic studies have shown that a high consumption of fruits and vegetables is associated with a decreased risk of human cancer at a number of common sites (Block *et al.*, 1992; Miller, 1990; Negri *et al.*, 1991; Steinmetz and Potter, 1991; Weisburger, 1991). A high consumption of fruits and vegetables may also be beneficial with respect to cardiovascular disease risk (Gey *et al.*, 1993; Gramenzi *et al.*, 1990; Hertog *et al.*, 1993; Palgi, 1981). The potential health benefits of fruits and vegetables have been attributed to the effects of specific components therein, i.e., vitamins, minerals, dietary fiber and a wide range of secondary metabolites (phytochemicals) responsible for characteristics such as color, flavor and taste. Several of these components have been hypothesized to exert an important influence on human physiologic status (Tomás-Barberán and Robins, 1997). One possible defense mechanism of fruits and vegetables is the antioxidant capacity of several components, for example carotenoids and vitamin C and E. Plasma carotenoids have been associated with a decreased risk of cancer (Astorg, 1997) and cardiovascular disease (Palace *et al.*, 1999). Two specific carotenoids, lutein and zeaxanthin, may be important for protecting the macula lutea in the eye (Eye Disease Case-Control Study Group 1992 and 1993). Another protective mechanism of fruit and vegetables could be the potential influence of folate on homocysteine metabolism. Observational epidemiologic research has shown that a high plasma concentration of homocysteine may be a risk factor for cardiovascular disease. Several intervention studies have shown that supplementation with folic acid is effective in reducing plasma homocysteine concentration (Brouwer *et al.*, 1999a; De Bree *et al.*, 1997; Ubbink *et al.*, 1994). Previous studies on increased fruit and vegetable consumption have been published, but these focused on specific nutrients or products. Zino *et al.* (1997) and Yeum *et al.* (1996) showed that plasma carotenoid and vitamin concentrations increased as a result of an increased intake of vegetables and fruits. However, fat intake, an important determinant of bioavailability of carotenoids, especially of lutein esters (unpublished observations), was not controlled in the study of Zino *et al.* (1997). Yeum *et al.* (1996) controlled the diet for fat, but selected specifically carotenoid-rich vegetables and fruits. In contrast, De Pee *et al.* (1995) suggested that β-carotene from vegetables may be poorly bioavailable in tropical diets. Brouwer *et al.* (1999b) concluded that consumption of foods rich in folate

(vegetables and citrus fruits) could improve folate and homocysteine status. The current recommendation of health authorities (Steinmetz and Potter, 1996) is to increase fruit and vegetable consumption in general (e.g., 'five a day'). This is the first study to investigate the effects of this recommendation in a mixed population. The present study investigated whether an increased intake of a mix of fruit and vegetables with a controlled Western diet had effects in human volunteers on plasma carotenoids, vitamin C, α-tocopherol, plasma homocysteine, vitamin B-12 and folate.

Materials and methods

Subjects

Subjects aged 40–60 y were recruited from the pool of volunteers of the Institute and through an advertisement in a local newspaper. They were preselected on the basis of a low habitual fruit and vegetable consumption (< 250 g/d) and a regular Dutch food intake pattern according to a questionnaire. The protocol was explained to the volunteers before they gave their informed consent. The major inclusion criteria were a body mass index [BMI; body weight (kg) / height^2 (m^2)] < 35 kg/m^2, alcohol consumption < 40 g/d ethanol (men) and 30 g/d ethanol (women), no use of vitamins or other food supplements, no metabolic or endocrine disease, no allergies to fruits and vegetables, not pregnant and/or lactating, no prescribed medication (except paracetamol and oral contraceptives) and a serum cholesterol concentration < 8 mmol/L. A total of 48 (24 men and 24 women) eligible volunteers entered the study; 12 smokers, 6 of each gender.

Study protocol

The study was performed according to the guidelines for Good Clinical Practice of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, and was approved by an external Medical Ethical Committee. We performed a 4-wk randomized, single blind, parallel, diet-controlled intervention study. By design, participants were stratified for gender and smoking habits. Further, several random (high/low) allocations were generated by a computer program. The random solution on the group level that yielded the most homogeneous age and BMI distribution was then selected. Volunteers (n = 24) received a diet low in fruits and vegetables (100 g/d; 'low'

group); another 24 volunteers received a diet high in fruits and vegetables (500 g/d; 'high' group) and also drank fruit juices (200 mL/d).

Food intakes were monitored every day by consumption of the evening meal under supervision at the Institute between 1700 and 1900h. Remaining parts of the dinner were weighed and recorded. The rest of the daily diet (breakfast and lunch) was handed out to the volunteers in a box after the contents were checked. When the subjects returned the next evening, the consumption of breakfast and lunch was controlled and recorded by checking the box. The volunteers had to complete a form with the questions 'Did you consume the total supplied amount of food today?' and 'Did you consume foodstuffs and drinks other then the provided food and drinks?'. The study was executed at the Department of Nutritional Physiology of TNO Nutrition and Food Research, Zeist from September 1997 to October 1997.

Diets

All volunteers received the same basic diet with different energy levels according to their need (7.7, 8.7, 9.7, 10.7, 11.7 and 12.7 MJ/d). The energy intake of breakfast, lunch and snacks differentiated the energy levels of the diets. The diets were controlled for energy, fat, protein and carbohydrates. During the study period, body weights were measured twice weekly before the evening meal. If the measured body weight deviated ≥ 1.5 kg compared with the reference body weight at day 0, the subjects were shifted to a diet one energy level higher or lower. Following the Dutch National Food Consumption Survey-2, fruits and vegetables normally consumed in the age group 40–60 y were selected. Table 1 shows the 7-day menu cycle of fruits and vegetables of the two diets.

Blood collection

On day 1 and 29, blood samples were collected from fasting subjects. For the analysis of carotenoids, vitamin C and α-tocopherol, blood was collected in tubes containing lithium heparin (Vacutainer systems, Becton Dickinson, Leiden, The Netherlands) and further prepared in yellow light. Within 30 min, the tubes were centrifuged (2000 × *g*, 10 min, 4°C). Metaphosphoric acid solution (50 g/L; J.T. Baker, Deventer, The Netherlands) was added to the plasma for the analysis of vitamin C. Samples were stored at –80°C until analysis. Blood was collected in tubes containing K_3EDTA (Vacutainer systems, Becton Dickinson) for the analysis

of vitamin B-12, folate and homocysteine. Within 15 min blood was centrifuged (2000 × *g*, 10 min, 4°C). Plasma samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Analyses were carried out after all samples were collected.

Biochemical analyses

Carotenoid profiles and α-tocopherol in plasma were quantified by HPLC using a modified version of a method described previously (Van Vliet *et al.*, 1991). After precipitation of proteins by ethanol (J.T. Baker), carotenoids and tocopherols were extracted from plasma by *n*-hexane (Merck, Darmstadt, Germany) and separated by HPLC (Hyperchrome column; Bisschof, Leonberg, Germany). Two absorbency detectors (ABI Analytical Kratos Division, South Yorkshire, UK) were used in series for the colorimetric determination of carotenoid profiles (450 nm) and α-tocopherol (286 nm). The CV was as follows: lutein, ≤ 16.6%; zeaxanthin, ≤ 36.8%; β-cryptoxanthin, ≤ 7.5%; lycopene, ≤ 9.7%; α-carotene, ≤ 23.4%; β-carotene, ≤ 6.3%; and α-tocopherol ≤ 3.2%.

Vitamin C in plasma containing metaphosphoric acid was quantified by HPLC using a modified version of a method previously described (Speek *et al.*, 1984). All ascorbic acid was oxidized to dehydro-L-ascorbic acid using ascorbate oxidase (Boehringer). Quinoxaline derivate was separated by reversed phase HPLC (Hyperchrome column, Bisschof) after condensation with 1,2-diaminobenzene (Merck). A fluorescence detector (Jasco, Tokyo, Japan) was used for the detection of vitamin C (CV ≤ 11.6%).

Vitamin B-12 and folate were quantified by competitive protein-binding assay (Simultrac Radioassay Kit Vitamin B-12 [^{57}Co] Folate [^{125}I], ICN Pharmaceuticals, ICN Biomedicals, Zoetermeer, the Netherlands), according to Dunn and Foster (1973), Lau *et al.* (1965) and Kolhouse *et al.* (1978). In summary, vitamin B-12 and folate in plasma were extracted by heat denaturation of the endogenous binding proteins. In the presence of potassium cyanide (ICN Biomedicals) cobalamins were converted to cyanocobalamin. To prevent oxidation of 5-methyltetrahydrofolic acid dithiotreitol (ICN Biomedicals) was added. After incubation with ^{57}CoB-12, ^{125}I folic acid, Intrinsic Factor (ICN Biomedicals) and α-lactoglobuline (ICN Biomedicals), the radioactivity was counted. The CVs were ≤ 11.6% and ≤ 13.0% for vitamin B-12 and folate, respectively.

Table 1 7-d menu cycle of fruits and vegetables (*g*) of the 'high' and 'low' groups during the dietary intervention period

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
High group dinner	french beans (140) red sweet pepper (20) mushrooms (20) onions (20) cucumber (40) tomatoes (35)	carrots (200) chicory (38) apple (20) orange (20)	red cabbage with apples (150) apple-sauce (50) lettuce (45) carrots (30)	cauliflower (200) cucumber (40) tomatoes (35)	sprouts (200) lettuce (50) red sweet pepper (25)	mushrooms (42) onion (30) lettuce (40) white cabbage (38) tomatoes (50) lettuce (63) corn (12)	endive (200) chicory (38) apple (20) orange (20)
High group during the day	orange (200) melon (100) orange juice (200mL)	orange (200) tangerine (2 × 90) apple juice (200 mL)	apple (150) apple (75) orange juice (200mL)	pear (160) kiwi (90) apple juice (200 mL)	apple (150) apple (75) orange juice (200 mL)	orange (200) tangerine (2 × 90) apple juice (200mL)	banana (165) grapes (100) orange juice (200 mL)
Low group dinner	french beans (35) red sweet pepper(5) mushrooms (5) onions (5)	carrots (50)	red cabbage with apples (50)	cauliflower (50) cucumber (25) tomatoes (25)	sprouts (50)	mushrooms (15) onion (8) white cabbage (15) red sweet pepper (13) lettuce (42) corn (8)	endive (50) chicory (25) apple (12) orange (13)
Low group during the day	apple (50)	tangerine (50)	orange (50)		orange (50)		

Total plasma homocysteine was determined by HPLC with fluorescence detection using a method previously described (Ubbink *et al.*, 1991). Before reversed-phase HPLC analysis, the plasma thiols were derivatized with ammonium 7-fluorobenzo-2-oxa-1,2-diazole-4-sulfonate (SBD-F), a thiolspecific fluorogenic probe that is commercially available. Retention of SBD-homocysteine was sensitive to pH, and a mobile phase pH of 2.1 ensured baseline separation of plasma thiols within 6 min. The CV was $\leq 4.5\%$.

Chemical analyses of diet

Total weekly high and low diets were mixed and homogenates were made. Because the meals differed in energy level according to the need of the volunteers, a high and a low diet with a calculated energy level of 10.7 MJ was used for analysis. The homogenates were used for the analysis of vitamin C, carotenoids, vitamin E, vitamin B-6, folate and total energy. Another part of the homogenate was freeze-dried and ground, using a strainer. This freeze-dried homogenate was used for the analysis of protein, fatty acids, carbohydrates, sugars, fiber and glucosinolates. Flavonoids analyses were carried out only in fruits and vegetables with a measurable amount of flavonoids. All of these analyses were performed using standard methods. References are available upon request.

Statistical analysis

Data analyses were performed using the statistical software package SAS/STAT (Version 6, SAS Institute, Cary, NC, General Linear Models procedure). First, for description of our data, baseline values were compared between groups (t test), and baseline and final values within groups were examined by a paired t test. Second, to quantify the unbiased overall effect of the intervention we used ANOVA to calculate the differences in final values of each variable between the low and high group after adjustment for gender, smoking habits and baseline plasma concentrations of each variable. Analysis of covariance was used for statistical testing. Data are reported as mean $\pm$ SD unless stated otherwise and $P < 0.05$ indicated significant difference.

Results

The groups did not differ in age, BMI, total plasma cholesterol, systolic and diastolic blood pressure at baseline and in the habitual daily intake of vegetables and fruits before the start of the study (Table 2).

Table 2 Characteristics of participants pre-study in the 'low' and 'high' groups[1]

	Intervention	
	'Low' group	'High' group
Age, y	50 ± 4.38	48.6 ± 5.63
Men / women, *n*	12 / 11	12 / 12
Smokers, %	26	25
Intake of vegetables and fruits, g/d	158 ± 54	162 ± 46
Body weight, kg	77.5 ± 11.8	79.5 ± 11.4
Body mass index, kg/m^2	25.2 ± 2.9	26.3 ± 3.3
Height, cm	175.2 ± 9.9	173.9 ± 7.5
Total plasma cholesterol, mmol/L	6.0 ± 0.9	5.9 ± 0.9
Systolic blood pressure, mmHg	128 ± 19	126 ± 20
Diastolic blood pressure, mmHg	78 ± 11	79 ± 14

[1] Values are means ± SD unless stated otherwise.

At baseline, plasma zeaxanthin concentrations were significantly different between the groups ($P < 0.05$). One participant in the low fruit and vegetable consumption group, a nonsmoking female, did not complete the study and was not replaced. Data for this subject were not used in the statistical analysis. The daily energy intakes of the low and high groups during the trial were 10.2 ± 1.6 and 10.1 ± 1.2 MJ, respectively. The total energy content of both diets was similar. In both diets the energy percentages (en%) from fat, protein and carbohydrate were near those recommended for protein (12–13 en%), carbohydrate (40–55 en%) and fat (30–35 en%). The amounts of vitamin C, total carotenoids, folate, glucosinolates and flavonoids were 1.7–, 3.6–, 0.7–, 5.3–, 4.0–fold higher, respectively, in the high diet than in the low diet. The amount of vitamin E and vitamin B-6 were comparable in the two diets (Table 3).

The overall compliance in both groups was good. The volunteers consumed 98.9 and 99.7% of the provided energy of the high and low diets, respectively. The

compliance of fruit and vegetable intake separately was 99.7% of the energy in the high group and 99.8% of the energy in the low group. The changes in body weight after 25 days in the high and low groups were −1.5 and −1.7%, respectively ($P < 0.05$). These small reductions in body weight did not differ between groups.

In the low group, the concentrations of the carotenoids decreased significantly by 31–67%, depending on the carotenoid (Table 4). Vitamin C concentrations decreased significantly by 13% ($P < 0.05$) and α-tocopherol concentrations decreased by 8% ($P < 0.05$) in this group. In the high group, there were significant increases in plasma concentrations of α-carotene (67%), vitamin C (50%) and β-cryptoxanthin (28%), ($P < 0.05$). Plasma lutein and β-carotene concentrations did not change, but plasma lycopene decreased by 56% ($P < 0.05$). Plasma α-tocopherol concentrations decreased by 11% ($P < 0.05$), similar to change in the low group.

After adjustment for gender, smoking habits and baseline plasma concentrations, the final plasma concentrations of the high in comparison with the low group were significantly higher for lutein +46% [95% confidence interval (CI) +28 to +64); β-cryptoxanthin, +128% (+98 to +159); lycopene, +22% (+8 to +37); α-carotene, +121% (+94 to +149); β-carotene, +45% (+28 to +62); and vitamin C, +64% (+51 to +77).

No significant changes were found in either group for folate and vitamin B-12 after 4 wk of dietary intervention. Plasma homocysteine concentrations decreased significantly by 10% ($P < 0.05$) in the high group (Table 5). Differences in final plasma levels between the high group and the low group ('high'–'low') were as follows: folate, +15% (+0.8 to +30; $P < 0.05$) and homocysteine and −11% (−18 to −4; $P < 0.05$). Plasma vitamin B-12 concentrations did not differ between the two groups.

Table 3 Daily intake of nutrients and energy during the dietary intervention period[1]

	Intervention	
Energy/nutrient	'Low' diet	'High' diet
Total energy, MJ	11.2	11.4
Protein, en%	14.6	14.9
Total fat, en%	32.7	29.9
Carbohydrate, en%	53.5	53.3
Saturated fatty acids, en%	14.4	13.1
Monounsaturated fatty acids, en%	7.5	6.8
Polyunsaturated fatty acids, en%	6.5	6.0
Fiber, g	47.9	56.2
Vitamin C, mg	65.0	172.5
Total carotenoids, mg	2.9	13.3
Lutein, mg	0.5	2.0
Zeaxanthin, mg	0.04	0.13
β-Cryptoxanthin, mg	0.04	0.27
Lycopene, mg	0.06	0.66
α-Carotene, mg	0.27	1.33
β-Carotene, mg	0.63	2.98
Vitamin E, mg	17.6	19.9
Vitamin B-6, mg	1.61	1.96
Folate, μg	131.1	228.1
Total glucosinolates, μmol	2.3	14.6
Total flavonoids, mg	0.75	3.74

[1]Values are based on the chemical analyses of weekly diets (see Materials and Methods).

Table 4 Effect of 100 g/d fruits and vegetables ('low' group) and 500 g/d fruits and vegetables ('high' group) on plasma carotenoids (*all*-trans isomers), vitamin C (ascorbic acid) and α-tocopherol concentrations in fasting humans during a 4-wk dietary intervention period[1,2]

	'Low' group		'High' group		
	Baseline	Change	Baseline	Change	'High' – 'Low' after study[3]
	μmol/L				
Lutein	0.35 ± 0.16	−0.12 ± 0.12	0.32 ± 0.14	0.02 ± 0.11	0.11 (0.07–0.14)*
Zeaxanthin	0.05 ± 0.04	−0.02 ± 0.02	0.04 ± 0.02	−0.004 ± 0.02	0.005 (−0.004–0.01)
β-Cryptoxanthin	0.13 ± 0.07	−0.04 ± 0.04	0.18 ± 0.20	0.05 ± 0.14	0.13 (0.11–0.16)*
Lycopene	0.52 ± 0.28	−0.35 ± 0.20	0.39 ± 0.26	−0.22 ± 0.18	0.04 (0.01–0.06)*
α-Carotene	0.06 ± 0.04	−0.02 ± 0.04	0.06 ± 0.02	0.04 ± 0.02	0.06 (0.04–0.06)*
β-Carotene	0.43 ± 0.22	−0.17 ± 0.13	0.37 ± 0.19	−0.02 ± 0.11	0.11 (0.07–0.15)*
α-Tocopherol	33.2 ± 7.2	−2.7 ± 4.9	33.2 ± 7.7	−3.7 ± 4.2	−1.0 (−3.4–1.3)
Vitamin C	51.0 ± 20.5	−6.7 ± 15.4	47.9 ± 16.9	24.0 ± 18.0	28.1 (22.3–34.0)*

[1] Values are means ± SD unless stated otherwise; [2] For some individual measurements, data are missing; [3] Difference in final (95% confidence interval), corrected for gender, smoking habit and baseline values in the 'high' *vs* 'low' group, covariance analyses; *$P < 0.05$.

Table 5 Effect of 100 g/d fruits and vegetables ('low' group) and 500 g/d fruits and vegetables ('high' group) on plasma vitamin B-12, folate and homocysteine concentrations in fasting humans during a 4-wk dietary intervention period[1,2]

	'Low' group		'High' group		
	Baseline	Change	Baseline	Change	'High' – 'Low' after study[3]
Vitamin B-12 pmol/L	251.5±121.8	−0.14 ± 40.7	270.8±85.2	−2.5 ± 29.7	0.02 (−19.5 – 19.6)
Folate nmol/L	13.2 ± 6.0	−0.96 ± 4.18	15.5 ± 10.2	0.14 ± 4.64	1.99 (0.11 – 3.87)*
Homocysteine µmol/L	13.8 ± 6.0	−0.56 ± 3.03	12.1 ± 3.6	−1.2 ± 2.1	−1.36 (−2.25 to −0.47)*

[1] Values are means ± SD unless stated otherwise. [2] For some individual measurements, data are missing. [3] Difference in final (95% confidence interval), corrected for gender, smoking habit and baseline values in the 'high' *vs* 'low' group, covariance analyses; *$P < 0.05$.

Discussion

The main findings of this study are that the consumption for 4 weeks of 500 g fruits and vegetables in comparison with 100 g fruits and vegetables resulted in significantly higher plasma carotenoids concentrations, including lutein (46%), β-cryptoxanthin (128%), lycopene (22%), α-carotene (121%), β-carotene (45%) and vitamin C (64%). Other important findings are an 11% lower plasma homocysteine concentration in the high group than in the low group. Furthermore, we found a small but significant plasma effect on folate (15%). Plasma concentrations of zeaxanthin, α-tocopherol and vitamin B-12 were not affected by the intervention, as could be expected. In this single blind trial, volunteers were carefully prestratified and were administered a habitual Dutch diet, which was completely controlled for energy, fat, protein and carbohydrate content. Reported compliance was very good because the major part of the diet containing fruits and vegetables was consumed under supervision. After the consumption for 4 weeks of 100 g fruits and vegetables plasma concentrations of carotenoids and vitamins decreased. The selected volunteers had an initial consumption of roughly ~150 g/d fruits and vegetables. The observed decrease of plasma concentrations is not surprising. Also, our brief questionnaire may have underestimated fruit and vegetable consumption somewhat.

Plasma lycopene concentrations of subjects that consumed 500 g of fruits and vegetables were 22% greater after 4 wk compared with subjects that consumed 100 g fruits and vegetables. However, lycopene concentrations decreased in both groups. In a study of Yeum *et al.* (1996), lycopene concentrations increased significantly after consumption of a diet with a high carotenoid content (of the 16 mg/d, 3.3 mg/d was lycopene). Possible explanations for the decrease of plasma lycopene during our study include a lower lycopene intake during the study in comparison with the prestudy period and a limited bioavailability of lycopene from the nonprocessed products (Van het Hof *et al.*, 1998). The preselection of volunteers was based on fruit and vegetable intake as a whole. The lycopene intake cannot be estimated from this questionnaire. In the prospective cohort study on diet and cancer in the Netherlands, the mean daily intake of lycopene was 1.05 ± 1.56 and 1.33 ± 1.88 mg/d for men and women, respectively (Goldbohm *et al.*, 1998). These daily intakes are ~0.4–0.7 mg/d higher that of the high diet. In a study of Van het Hof *et al.* (1999), plasma concentrations of lycopene decreased by ~38% after consumption of 490 g/d vegetables with a lycopene content even higher (1.1 mg/d) then that of our high diet.

Concentrations of β-carotene showed a difference of +45% in the high group in comparison with the low group. However, the concentrations remained more or less stable in the high group and decreased significantly in the low group. Zino *et al.* (1997) performed a trial in which the intervention group was asked to increase their fruit and vegetable consumption to eight servings (~1000 g) per day. The plasma concentration of β-carotene was higher after 4 weeks compared with the present trial, but the β-carotene content of the intervention diet in the trial of Zino *et al.* (1997) was ~1.5 mg/d higher than in our trial. Also, subjects in the Zino study continued to consume their freely chosen habitual diet, including processed foods.

No difference between the changes in plasma zeaxanthin and α-tocopherol concentrations were observed between the low and high group. The CV for zeaxanthin measurements could be the reason for the lack of a significant difference. The daily dietary intake of vitamin E was comparable between the groups because fruits and vegetables do not contain a high amount of vitamin E. Therefore, no major differences between the diet treatments would be expected.

The strength of this study in comparison with previously described trials is the completely controlled intervention period for energy, fat, protein and carbohydrate

intake and the good compliance. Therefore, effects on plasma (fat-soluble) vitamins and carotenoids can be attributed to the differences in fruit and vegetable consumption alone. We examined the effects of increased fruit and vegetable consumption as a whole and not an increase of a specific micronutrient and/or specific fruit or vegetable as is to be expected in a population.

In this study the daily low diet contained 1.6 mg vitamin B-6 and 131 μg folate, whereas the daily high diet contained 2.0 mg vitamin B-6 and 228 μg folate. These differences in intake could theoretically not be expected to have a major influence on plasma homocysteine. Minimal doses of supplemented folic acid of 250 μg/d with a high bioavailability have been reported to lower homocysteine concentrations (Brouwer *et al.*, 1999a). Food contains folate polyglutamate derivatives, which are hydrolyzed to monoglutamate forms in the gut before absorption. Therefore the bioavailability of food folate is lower than the bioavailability of folic acid supplements. In the intervention trial of Brouwer *et al.* (1999b) folate status was improved by 7 nmol/L and homocysteine concentrations were decreased by 2 μmol/L after consumption of vegetables and citrus fruits high in folate (560 μg/d). In comparison, the high diet in our trial contained 228 μg/d. During the study the volunteers consumed a normal Dutch diet, not a diet with vegetables and fruits high in folate concentration. Although we can only speculate here, our results suggest that other components in fruits and vegetables may influence plasma homocysteine.

The biological relevance of an 11% lower plasma homocysteine concentration, ~1.4 μmol/L, is not clear, but observational epidemiologic studies have found an inverse relationship between plasma homocysteine and cardiovascular disease. Thus, it is advisable to increase fruit and vegetable consumption, which could lower homocysteine concentrations.

In conclusion, our study suggests that a 4-week period of 500 g/d fruit and vegetable consumption in comparison with 100 g/d of fruits and vegetables with a diet controlled for energy, fat, protein and carbohydrates significantly increases plasma carotenoids, vitamin C, folate and decreases homocysteine concentrations, but has no effect on zeaxanthin, vitamin B-12 and α-tocopherol plasma concentrations. Extra consumption of fruits and vegetables (400 g) and 200 mL/d fruit juices with an equal fat consumption could increase plasma carotenoid and vitamin concentrations by 22-128% and influence plasma folate and homocysteine.

Plasma homocysteine (in addition to carotenoids) can be an important indicator of fruit and vegetable intake and is responsive to changes in the intakes of these foods.

Whether changes in plasma concentrations of vitamins and carotenoids have an effect on disease risk remains to be established. High plasma homocysteine concentrations are considered to be a risk factor for cardiovascular disease (Hankey and Eikelboom, 1999). This is the first trial that shows that a mix of fruits and vegetables, with a moderate folate content, will decrease plasma homocysteine levels.

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Chapter 8

Fruit and vegetables and cardiovascular risk profile: a diet controlled intervention study

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Abstract

Objective: To evaluate the effect of fruit and vegetables consumption on markers of risk for cardiovascular disease.

Design: Randomized, diet controlled, parallel study.

Subjects: Forty-eight apparently healthy (40–60 y) volunteers with a low usual consumption of fruits and vegetables. Forty-seven of them completed the study.

Interventions: During 4 weeks 24 volunteers consumed a standardized meal, consisting of 500 g/d fruit and vegetables and 200 mL/d fruit juice ('high' group) and 23 volunteers consumed 100 g/d fruit and vegetables ('low' group) with an energy and fat controlled diet.

Results: Final total cholesterol was 0.2 (95% CI −0.5 – 0.03 mmol/L) lower in the high group than in the low group ($P > 0.05$). Final fibrinogen and systolic blood pressure were 0.1 (−0.1 – 0.4) g/L and 2.8 (−2.6 – 8.1) mmHg higher in the high group than in the low group ($P > 0.05$), respectively. Also, other final serum lipid concentrations, diastolic blood pressure and other hemostatic factors did not differ between both groups.

Conclusions: This was a small randomized well-controlled dietary intervention trial of short duration with a considerable contrast in fruit and vegetable consumption. No effects on serum lipids, blood pressure and hemostatic variables were observed.

Introduction

Cardiovascular disease is the main cause of mortality in The Netherlands and other Western countries. Risk factors include smoking, high blood pressure, high serum cholesterol levels and high levels of homocysteine. The development of atherosclerosis is multifactorial and relevant pathogenic processes include oxidative damage of low density lipoprotein (LDL) cholesterol (Steinberg *et al.*, 1989).

Observational epidemiological studies have shown that a high consumption of fruit and vegetables may be associated with a decreased risk of cardiovascular disease (Ness and Powles, 1997). Fruit and vegetables contain vitamins, fibers and other active compounds. These phytochemicals have different mechanisms of action. For example, they may modulate cholesterol metabolism, reduce blood pressure and have antioxidant effects (Lampe, 1999). These effects have been investigated in cell cultures and animal experiments.

Fruit and vegetables consumption may lower the concentration of plasma cholesterol because of high soluble dietary fiber, such as pectin (Stasse-Wolthuis *et al.*, 1980). Dietary fiber and minerals may explain why a diet rich in fruit and vegetables may reduce blood pressure (Appel *et al.*, 1997). Another additional mechanism is the influence of fruit and vegetables on blood platelet aggregation (Lampe, 1999). Furthermore, folate-rich fruit and vegetables could decrease homocysteine concentrations (Brouwer *et al.*, 1999).

The current recommendation of health authorities is to increase fruit and vegetables consumption in general (e.g. 'five a day'; Steinmetz and Potter, 1996). This results in more than 400 g fruit and vegetables per day. So far, experimental studies have not evaluated this recommendation, but have used either especially selected vegetables (Yeum *et al.*, 1996) or very high quantities (approximately 7 servings/day) of fruit and vegetables (Zino *et al.*, 1997). Moreover, the association between health and vegetable consumption in epidemiological studies could be confounded by a generally healthier lifestyle and/or lower intake of energy and fat of high vegetable consumers (Willett, 1998). Further experimental studies in humans are therefore necessary to demonstrate that potential beneficial effects are due to a high, but realistic consumption of fruit and vegetables *per se*. Therefore we performed a human intervention trial with a diet controlled for energy, fat, carbohydrates and protein. This is the first study that investigates the effects of a

fruit and vegetables consumption, approximately compatible with the current dietary recommendation in a study group. We compared an intake of 500 g/d fruit and vegetables and 200 mL/d fruit juice with 100 g/d fruit and vegetables, which will reflect a difference of 400 g/d fruit and vegetables and 200 mL/d fruit juice. We evaluated effects on serum lipids, blood pressure and indicators of coagulation and fibrinolysis.

Subjects and methods

Study design

The design of this randomized diet-controlled human intervention study was described previously (Broekmans *et al.*, 2000). In summary, subjects aged 40–60 y were recruited both from the pool of volunteers of the Institute and through an advertisement in a local newspaper. All participants had low habitual fruit and vegetable consumption (< 250 g/d) according to a questionnaire and did not use vitamins or other food supplements. By design, the volunteers were stratified for gender and smoking habits. Further, several random group allocations were generated by a computer program. The random solution on the group level that yielded the most homogeneous age and BMI distribution was then selected. The volunteers consumed either a diet low in fruits and vegetables (100 g/d; 'low' group) or a diet high in fruit and vegetables (500 g/d and 200 mL fruit juice/d; 'high' group). The diets contained a variety of fruit and vegetables and were controlled for energy, fat, protein and carbohydrates. Food intake was monitored every day by consumption of the evening meal under supervision at the Institute between 1700 and 1900h. The rest of the daily diet (breakfast and lunch) was handed out to the volunteers in a box after the contents were checked. Fasting blood samples were collected before and after the 4 weeks of treatment for analyses of serum lipids and blood coagulation/fibrinolysis parameters. During the study blood pressure and body weight were measured. The study was executed at the Department of Nutritional Physiology of TNO Nutrition and Food Research, Zeist from September 1997 to October 1997. The study was performed according to the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines for Good Clinical Practice and was approved by a Medical Ethical Committee.

Blood parameters

To analyze cholesterol and triacylglycerols levels blood was collected in tubes with clot activating silica particles. Approximately 30 min after collection, tubes were centrifuged for 10 min, 3000 × *g*, 4°C and stored at –20°C until the analysis. Serum triacylglycerols, total cholesterol and HDL cholesterol concentrations were analyzed with a commercially available kit (Roche, Almere, The Netherlands). Serum LDL cholesterol concentration was calculated using the formula by Friedewald *et al.* (1972): [LDL] = [total cholesterol] – [HDL] – 0.42 × [triacylglycerols].

For the analysis of fibrinolytic/coagulation factors, blood was drawn into ice-chilled tubes and centrifuged for 20 min at 2000 × *g* and 4°C. Plasma was collected, immediately frozen by use of liquid nitrogen and stored at –80°C until analysis.

For measuring tissue-type plasminogen activator (tPA-activity), blood was collected in Stabilyte tubes. tPA activity was measured by a biological immunoassay according to Meijer *et al.* (1992). In summary, the active tPA was isolated from plasma by binding it to a microtiter-plate-fixed monoclonal antibody for tPA that binds tPA so that the active centre remained effective. After washing, a mixture of plasminogen and chromogenic plasmin substrate was added and the conversion of plasminogen to plasmin by the bound tPA was recorded. Both the intra-assay CV and inter-assay CV were < 10%.

To determine tPA antigen, F1+F2 and D-dimers blood was collected in tubes containing CTAD buffer. tPA antigen level was measured with an enzyme-linked immunosorbent assay (ELISA) using the Imulyse t-PA kit of Biopool (Ranby *et al.*, 1986). The intra-assay CV was < 10% and the inter-assay CV was < 12%. The amount of prothrombin fragment 1.2 (F1+F2) was detected by Sandwich ELISA, using Enzygnost® F1+2 micro method from Dade-Behring, Marburg, Germany (Pelzer *et al.*, 1991). F1+F2 in the sample was first bound to a solid phase rabbit anti F1+F2 antibodies coated on polystyrene Microelisa strips. After the second reaction with a rabbit antibody labelled with horseradish peroxidase, the color was spectrophotometrically determined. The inter- and intra-assay CV were < 10%.

D-dimers were measured, using the Enzygnost® micro method from Dade-Behring, Marburg, Germany (Estivals *et al.*, 1991). First, D-dimers in the samples were bound to a solid phase murine anti D-dimer antibody coated on polystyrene

Microelisa strips. Subsequently, a second antibody was bound to the immobilized antibody antigen complex. This second antibody was a rabbit antibody directed against fibrinogen, labelled with horse-radish peroxidase. The color was spectrophotometrically determined. The intra- and inter-assay CV were < 10%.
Fibrinogen was detected according to Clauss (1957) using the STA fibrinogen kit on a STA analyser. The diluted plasma was supplemented with a fixed amount of thrombin. This induced the conversion of fibrinogen to insoluble fibrin and resulted in clot formation. The speed of clot formation was dependent upon the concentration of fibrinogen in the sample. The STA analyser registered the clot formation as the viscosity of the solution. The intra-assay CV was < 5% and the inter-assay CV < 7%.
For measuring the compliance of the diets, plasma vitamin C, folate and carotenoids were determined. The methods are previously described (Broekmans *et al.*, 2000). In summary, carotenoid profiles and vitamin C in plasma were quantified by HPLC. Folate was quantified by competitive protein-binding assay.

Blood pressure
During the study blood pressure was measured eight times: two times on days 1 and 28 in the evening and twice at day 2 and 29 in the morning. Measurements of day 1 and 2 were averaged, as were the measurements of day 28 and 29. Both systolic and diastolic blood pressure and heart rate were measured oscillometrically by a Boso Oscillomat in a sitting position in the right arm after at least 5 min rest and recorded in mmHg and beats/minute respectively.

Chemical analyses of the diets
Total weekly 'low' and 'high' diets were mixed and homogenates were made. We used diets with an energy level of 10.7 MJ for the analysis. We measured vitamin C, carotenoids, vitamin E, vitamin B-6, folate and total energy in the homogenates. A freeze-dried homogenate was used to analyze protein, fatty acids, carbohydrates, sugars, fiber and glucosinolates. Flavonoids analyses were carried out only in fruit and vegetables with a measurable amount of flavonoids. All these analyses were performed using standard methods. References are available upon request.

Statistical analysis

Data analyses were performed using the statistical software package (SAS/STAT Version 6, SAS Institute, Cary, NC, General Linear Models procedure). First, for description of our data, baseline values were compared between groups (t test), and baseline and final values within groups were examined by a paired t test. Second, to quantify the unbiased overall effect of the intervention, we used ANOVA to calculate the differences in final values of each variable between the 'low' and 'high' group after adjustment for gender, smoking habits and baseline values of each variable. Analysis of covariance was used for statistical testing. Data are reported as mean ± SD unless stated otherwise and $P < 0.05$ indicates significant difference. *A posteriori* power analysis showed that the number of subjects was sufficient to observe a 0.45 mmol/L difference in final total cholesterol, a 0.45 g/L difference in final fibrinogen concentrations and a 9.4 mmHg difference in final systolic blood pressure with a power of 0.9 and α is 0.05 (two-sided). The analysis of covariance revealed minor deviations from normality assumptions for some variables. For these variables, analyses were repeated using logarithmically transformed data. These log transformed analyses yielded results that are virtually identical to the untransformed data-analysis as presented here.

Results

The groups were similar in age, BMI and in the mean habitual daily intake of vegetables and fruit before the start of the study (Table 1).

Table 1 Characteristics of participants pre-study in the 'low' group and the 'high'group[1]

	Intervention	
	'Low' group	'High' group
Age, y	50 ± 4.38	48.6 ± 5.63
Men / women, *n*	12 / 11	12 / 12
Smokers, nonsmokers, *n*	6/17	6/18
Intake of vegetables and fruits, g/d	158 ± 54	162 ± 46
Body weight, kg	77.5 ± 11.8	79.5 ± 11.4
Body mass index, kg/m^2	25.2 ± 2.9	26.3 ± 3.3
Height, cm	175.2 ± 9.9	173.9 ± 7.5

[1] Values are means ± SD unless stated otherwise.

One participant of the 'low' fruit and vegetables consumption group, a non-smoking female, did not complete the study and was not replaced. Data for this non-complier are not used in the statistical analysis. The mean daily provided energy intake of the 'low' and 'high' groups during the trial were 10.2 ± 1.6 MJ and 10.1 ± 1.2 MJ, respectively.

Table 2 gives the analytical results for the diets designed at 10.7 MJ level. The actual energy level was slightly higher, but the total energy content of both diets was similar. In both diets the energy percentages from fat, protein and carbohydrate were near the recommended energy percentages (en%) for protein, carbohydrate and fat.

The overall compliance in both groups was good. The volunteers consumed 98.9% of the provided energy of the 'high' diet and 99.7% of the provided energy of 'low' diet. After 25 days, the mean changes in body weight in the 'high' and 'low' groups were similar. The compliance of fruit and vegetable intakes separately was 99.7% of the energy in the 'high' group and 99.8% of the energy in the 'low' group.

Plasma concentrations of vitamin C, folate, carotenoids were also measured, the results of which have been presented elsewhere (Broekmans *et al.*, 2000). Briefly, significant higher concentrations were observed in the 'high' group in comparison with the 'low' group for vitamin C (64%), folate (15%) and carotenoids, for example β-carotene (45%).

Baseline values and changes in serum lipids, hemostatic variables and blood pressure per group are shown in Table 3.

Table 2 Daily intake of nutrients and energy during dietary intervention period[1]

	Intervention	
Energy/nutrient	'Low' diet	'High' diet
Total energy, MJ	11.2	11.4
Protein, en%	14.6	14.9
Total fat, en%	32.7	29.9
Carbohydrate, en%	53.5	53.3
Saturated fatty acids, en%	14.4	13.1
Monounsaturated fatty acids, en%	7.5	6.8
Polyunsaturated fatty acids, en%	6.5	6.0
Fiber, g	47.9	56.2
Vitamin C, mg	65.0	172.5
Total carotenoids, mg	2.9	13.3
Lutein, mg	0.5	2.0
Zeaxanthin, mg	0.04	0.13
β-Cryptoxanthin, mg	0.04	0.27
Lycopene, mg	0.06	0.66
α-Carotene, mg	0.27	1.33
β-Carotene, mg	0.63	2.98
Vitamin E, mg	17.6	19.9
Vitamin B-6, mg	1.61	1.96
Folate, μg	131.1	228.1
Total glucosinolates, μmol	2.3	14.6
Total flavonoids, mg	0.75	3.74

[1] Values are based on the chemical analyses of weekly diets (see method section).

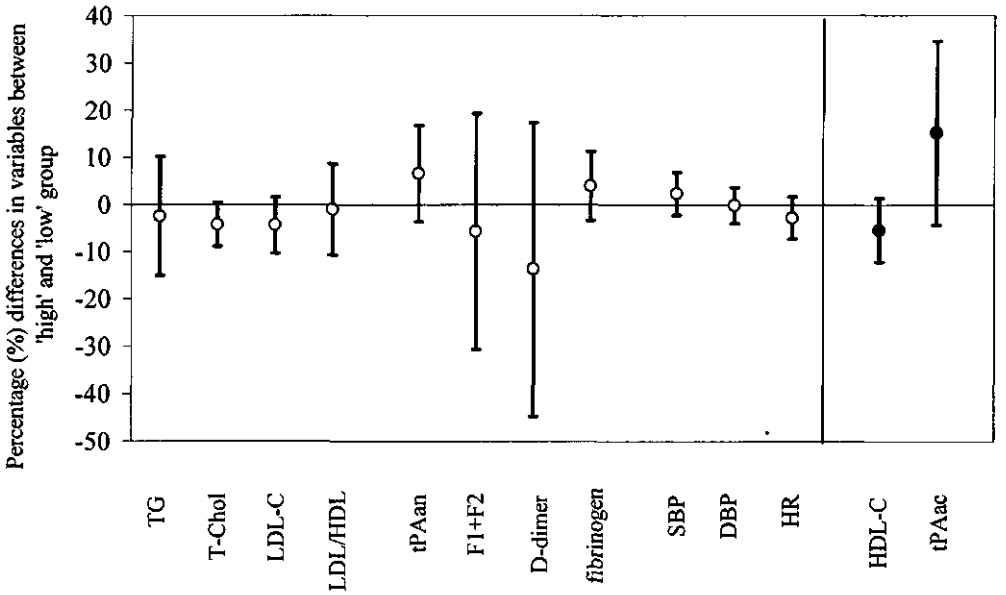
Table 3 Baseline values and changes of markers of cardiovascular risk profile during a 4 week trial in fasting human volunteers assigned to either the 'low' (n = 23) or 'high' (n = 24) fruit and vegetables group[1,2]

	'Low' group		'High' group		
	Baseline	Change	Baseline	Change	'High' – 'Low' after study[3]
Serum lipids					
Triacylglycerol (mmol/L)	1.5 ± 1.2	−0.1 ± 0.4	1.8 ± 1.1	−0.2 ± 0.5	−0.04 (−0.2–0.2)
Total cholesterol (mmol/L)	5.8 ± 1.0	−0.5 ± 0.5*	5.9 ± 1.2	−0.7 ± 0.7*	−0.2 (−0.5–0.03)
HDL cholesterol (mmol/L)	1.5 ± 0.3	−0.1 ± 0.2*	1.4 ± 0.5	−0.2 ± 0.2*	−0.1 (−0.2–0.02)
LDL cholesterol (mmol/L)	3.7 ± 0.9	−0.3 ± 0.5*	3.7 ± 1.1	−0.5 ± 0.6*	−0.2 (−0.4–0.1)
LDL/HDL	2.7 ± 0.9	0.02 ± 0.6	2.8 ± 1.2	−0.1 ± 0.6	−0.03 (−0.3–0.3)
Hemostatic variables					
tPA antigen (ng/mL)	9.4 ± 3.5	−1.0 ± 1.7*	10.6 ± 3.3	−0.6 ± 1.7	0.6 (−0.3–1.5)
tPA activity (IU/mL)	0.7 ± 0.3	0.1 ± 0.2*	0.5 ± 0.3	0.2 ± 0.2*	0.1 (−0.03–0.2)
F1+F2 (nmol/L)	1.7 ± 0.9	−0.3 ± 0.9	2.0 ± 1.5	−0.7 ± 1.5*	−0.1 (−0.4–0.3)
D-dimer (µg/L)	16.9 ± 10.9	2.7 ± 12.7	21.1 ± 16.0	0.9 ± 10.0	−3.0 (−9.6–3.7)
Fibrinogen (g/L)	3.1 ± 0.6	0.3 ± 0.5*	3.1 ± 0.5	0.5 ± 0.5*	0.1 (−0.1–0.4)
Blood pressure					
Systolic blood pressure (mmHg)	126 ± 15	−7.7 ± 8.4*	129 ± 16	−5.8 ± 11.2*	2.8 (−2.6–8.1)
Diastolic blood pressure (mmHg)	80 ± 10	−3.9 ± 6.1*	81 ± 10	−4.4 ± 6.4*	−0.1 (−3.1–2.8)
Heart rate (beats/min)	78 ± 10	−1.0 ± 6.0	77 ± 10	−2.9 ± 5.6*	−2.1 (−5.6–1.3)

[1] Values are means ± SD unless stated otherwise. [2] For some individual measurements data are missing. [3] Difference in final values (95% CI), corrected for gender, smoking habit and baseline values in the 'high' *vs* 'low' group, covariance analyses * Significant change within group, $P < 0.05$.

At baseline, only tPA activity values were higher in the 'low' group than in the 'high' group ($P < 0.05$). All other values were similar. Figure 1 shows the percentage differences plus 95% CI between the 'high' and 'low' fruit and vegetable group for serum lipids, hemostatic variables, blood pressure, heart rate, respectively. The variables are subdivided in variables where a decrease would be desirable (triacylglycerols, total cholesterol, LDL cholesterol, LDL/HDL ratio, tPA antigen, F1+F2, D-dimers, fibrinogen, systolic blood pressure, diastolic blood pressure, heart rate) and variables where an increase would be desirable (HDL-cholesterol and tPA activity).

Figure 1 Percentage differences (95% CI) in serum lipids, hemostatic variables and blood pressure between 500 g/d fruit and vegetables + 200 ml fruit juice/d and 100 g/d fruit and vegetables after 4 weeks, subdivided in variables with a desirable decrease (open dots) and a desirable increase (filled dots).



TG: serum triacylglycerols; T-Chol: total serum cholesterol; LDL-C: serum LDL cholesterol; LDL/HDL: serum LDL cholesterol/ HDL cholesterol; tPAan: plasma tPA antigen; F1+F2: plasma prothrombin fragment 1.2; D-dimer: plasma D-dimers; fibrinogen: plasma fibrinogen; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; HDL-C: serum HDL cholesterol; tPAac: plasma tPA activity.

Total cholesterol, HDL cholesterol and LDL cholesterol decreased both in the 'high' and in the 'low' group. LDL/HDL ratios remained stable in both groups. After adjustment for baseline values, gender and smoking, there were no significant differences in the final serum concentrations between the 'high' in comparison with the 'low' group for triacylglycerols, total cholesterol, HDL cholesterol, LDL cholesterol and LDL/HDL ratio. Blood pressure and heart rate decreased in both groups. We could not demonstrate intervention effects on blood pressure and hemostatic variables.

Discussion

This small randomized well-controlled dietary intervention trial of short duration shows that increase with 400 g/d of fruit and vegetable and 200 mL/d fruit juice consumption did not materially affect serum lipids, blood pressure and hemostatic variables in apparently healthy volunteers.

Elevated serum cholesterol, triacylglycerols, LDL cholesterol and reduced HDL cholesterol are risk factors for cardiovascular disease (Wald and Law, 1995; Criqui, 1998; Werner and Pearson, 1998; Jacobs *et al.*, 1990). Although there was a tendency for the LDL cholesterol to be lower in the 'high' group (–4%) than in the 'low' group, there was a similar lower concentration of HDL cholesterol (–5%). Both groups lost weight, but this was not different between the two groups. Weight loss could influence the serum levels of total cholesterol, LDL and HDL cholesterol. However, we did not find significant correlations between weight loss during the study and changes in total cholesterol, HDL and LDL cholesterol. The ratio LDL/HDL is more important as a cardiovascular risk indicator than individual LDL or HDL measurements. This ratio remained stable during the intervention of 400 g fruit and vegetables and 200 mL fruit juice consumption.

During the intervention in this study, serum lipids decreased in the 'high' as well as in the 'low' group. We did not measure energy and fat content of the habitual diet before the start of the study, but we may speculate that the 4-week controlled diet provided less energy and fat than the habitual diet consumed before the start of the study. Our results with regard to serum lipids are in agreement with other studies. In a diet-controlled study by Stasse-Wolthuis *et al.* (1980), total cholesterol decreased non-significantly by only 0.17 mmol/L with the diet of fruit and vegetables (1170 g/d) after 5 weeks. Two possible factors that could influence the

serum lipids are dietary fiber and dietary fat (Anderson, 1985). In our study dietary fat was controlled, whereas the fiber content was almost the same in both groups (Table 2). Zino *et al.* (1997) compared a recommendation of approximately 7 servings/d with a habitual diet of 2 servings/d in an 8-week trial. The differences between groups for total cholesterol, LDL, HDL and triacylglycerols were minor and lower than in our study.

It is possible to improve serum lipid profiles with a dietary intervention during 4 weeks. For example, Kurowska *et al.* (2000) reported that consumption of 750 mL/d but not 250 or 500 mL/d orange juice significantly increased HDL cholesterol and reduced the LDL/HDL cholesterol ratio in hypercholesterolemics. LDL cholesterol concentrations were not influenced. Potentially beneficial components in citrus juice could be folate, flavonoids and vitamin C. The content of folate and flavonoids in our 'high' and 'low' diets were not very high. However, vitamin C intake was much higher in the 'high' group and serum vitamin C was approximately 30 μmol/L higher. In a cross-sectional analysis (Ness *et al.*, 1996), plasma vitamin C was not correlated with serum total cholesterol or LDL cholesterol in men and women. In women, higher plasma vitamin C was associated with higher HDL cholesterol and lower triacylglycerols concentrations. Our trial could not confirm this association of vitamin C in serum with HDL cholesterol.

Our study does not show a clear-cut beneficial effect of fruits and vegetables on hemostasis. However, our study was not specifically designed to test the effect of fruit and vegetables consumption on hemostatic variables. Evidence from large observational epidemiological studies links hemostatic variables to the risk of cardiovascular disease (Koenig, 1998; Kannell *et al.*, 1987; Juhan-Vague, 1996; Rugman *et al.*, 1994; Iacoviello *et al.*, 1998). In our study, we used fibrinogen and prothrombin activation peptide fragment F1+2 as markers of coagulation (Rugman *et al.*, 1994); and tPA activity and tPA antigen as markers of fibrinolysis (Koenig, 1998) and D-dimers as a marker of fibrin turnover. It is thought that hemostatic factors could be influenced by changes in diet. Famodu *et al.* (1999) reported a decreased fibrinolytic activity, including higher fibrinogen concentrations in a non-vegetarian group in comparison with vegetarian and semi-vegetarian groups. This difference is possibly attributed to the high consumption of dietary fiber because of availability of a large group of fresh vegetables in their diet. Ideally, for fruit and vegetables to positively influence hemostatic function, they should decrease tPA

antigen, F1+F2, fibrinogen and increase concentrations of tPA activity. In this study we did not see such a consistent beneficial pattern. We did observe a positive tendency for increased tPA activity and decreased F1+F2, but none of the effects are statistically significant. On the other hand, the non-significant shifts in tPA antigen and fibrinogen concentrations were not in line with the possible positive effects of fruit and vegetable consumption on hemostatic variables. Further research is necessary to investigate the mechanisms through which fruit and vegetables could influence the blood platelet aggregation, blood coagulation and fibrinolysis.

This study did not find an effect on blood pressure. In contrast, in a previous study of Appel *et al.* (1997) systolic blood pressure was 2% lower in a fruit and vegetable group in comparison with a control group; diastolic blood pressure was 1.3% lower. Furthermore, randomized controlled trials have confirmed that a vegetarian diet, including a high amount of fruit and vegetable consumption could also lower blood pressure (Rouse *et al.*, 1983; Margetts *et al.*, 1986). Our study did not have sufficient statistical power to pick up such small differences. As the confidence intervals for blood pressure overlap with the effect observed in the trial reported by Appel *et al.* (1997), the results cannot exclude a modest reduction in blood pressure. According to the lowest confidence limits, we suggest that the effect of our fruit and vegetable intervention was less than 2% and 4% for systolic and diastolic blood pressure, respectively. The absence of a significant difference between the groups in our study could thus be explained by number of participants or the duration of the study, which was not specifically designed to test a blood pressure hypothesis. The composition of the diets however may be a more likely explanation. The differences in the amount of potassium between the 'low' and 'high' vegetable and fruit group were much higher in the trial of Appel *et al.* (1997), 1700 and 4700 mg in both diets respectively in comparison with the amounts in our trial, 3228 and 4062 mg per day. Vitamin C is another compound that could have an effect on blood pressure (Ness and Sterne, 2000). In our trial, the 'high' group had much higher intakes of vitamin C (ca. 173 *vs* 65 mg) in comparison to the 'low' group, but no effects on blood pressure were found. Trials on vitamin C reported so far are too small to provide conclusive evidence of an effect of vitamin C on blood pressure. In addition, these trials used vitamin C supplementation in very high amounts (> 400 mg/d).

Conclusions

This small randomized well-controlled dietary intervention trial of short duration with a considerable contrast in fruit and vegetable consumption shows no effect on the ratio LDL/HDL, although there was a non-significant tendency for both HDL and LDL cholesterol to decrease. Blood pressure and hemostatic variables were not influenced by fruit and vegetable consumption. We previously reported lower final plasma homocysteine concentrations in the 'high' group than in the 'low' group from the same study. The results presented now suggest that there is a lack on effect on other cardiovascular risk factors.

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Chapter 9

General discussion

The main objective of the studies described in this thesis was to evaluate the potential health effects of carotenoids in normal physiological ranges in relation to eye, skin and cardiovascular health. In an observational study we evaluated the associations between serum carotenoid status in relation to biomarkers of eye, skin and cardiovascular health. The observational results were obtained from the baseline of an intervention study designed to evaluate the effects of decreased carotenoid concentrations, as caused by sucrose polyesters (SPE) on the same biomarkers. In a second intervention study, we evaluated the effects of a high but realistic intake of fruit and vegetable on serum carotenoid concentrations and biomarkers of cardiovascular health.

As mentioned in the introduction, in the causal pathway between diet and disease, one may distinguish biomarkers of exposure/internal dose, biomarkers of target function/biological response and intermediate endpoints, the latter two designated as biomarkers of enhanced function and biomarkers of reduced risk (Diplock *et al.*, 1999). In the relation between diet and disease two aspects are important: (1) the potential of diet: which food components affect the biomarker and may thus reduce risk? (2) the validity of the biomarker: does a change in the biomarker reflects a change in risk?

In this discussion we will discuss first the effects of intake of SPE and a high but realistic consumption of fruit and vegetables, that is related to carotenoid status in a normal range on biomarkers of enhanced function or reduced risk. Second we will discuss the predictive value of the biomarkers used in our studies and areas for future research are given.

Main findings: effects of carotenoids in the normal physiological range on biomarkers

In this discussion we will first look at eye health, then at skin health and finally at cardiovascular health. The main findings of the studies in this thesis are presented in Table 1.

Table 1 Studies described in this thesis and their main findings

Observational study

Chapter	*Subjects*	*Biomarkers of exposure/ internal dose*	*Biomarkers of target function/biological response/ intermediate endpoint*	*Associations with biomarkers per standard deviation (SD) of serum carotenoids or the difference between the 10th and 90th percentile. All associations are statistically significant ($P < 0.05$) unless mentioned otherwise*
2	376	Serum and adipose tissue lutein	Macular pigment (MP) density	**Men**: 1 SD increment in serum lutein is associated with 13% higher MP density; 1 SD increment in adipose tissue lutein is associated with 11% higher MP density **Women**: no associations
3	376	Serum, adipose tissue lutein MP density	Lens density	No associations between serum and adipose tissue lutein and lens density after adjustment for age 1 SD increment in MP density is associated with 2.7% lower lens density
4	335	Serum carotenoids	Minimal erythema dose (MED)	**Dark skin**: 1 SD increment in lutein is associated with 5.8% higher MED ($P = 0.04$). All other carotenoids are positively associated ($P > 0.05$) **Fair skin**: 1 SD increment in lutein is associated with 5.3% lower MED ($P = 0.06$). All other carotenoids are inversely associated ($P > 0.05$)
5	379	Serum carotenoids	Flow mediated vasodilation (FMD), intercellular adhesion molecule (sICAM-1), C-reactive protein (CRP), fibrinogen, leucocytes	sICAM-1: 7% lower in 90th percentile than 10th percentile of lutein and lycopene FMD: 14% higher by an increment of 1 SD in zeaxanthin CRP and leucocytes: 41% and 11% lower in 90th percentile than 10th percentile of β-carotene No associations with fibrinogen

Intervention studies

Chapter	*Subjects*	*Treatments*	*Duration*	*Serum carotenoids in comparison with control group*	*Effect on biomarkers*
6	341	0, 7, 10, 17 g/d sucrose polyesters	52 weeks	Lipid standardized 7 g/d: 0.2 – 21% ↓ 10 g/d: 11 – 29% ↓ 17 g/d: 13 – 33% ↓	No effect on oxidative damage biomarkers, MP density, MED and FMD
7/8	47	500 g/d fruit and vegetables + 200 mL fruit juice *vs* 100 g/d fruit and vegetables	4 weeks	Carotenoids: 22 – 128% ↑ Vitamin C: 64% ↑; Folic acid: 15% ↑	Homocysteine: 11% ↓ No effect on blood pressure, serum lipids, fibrinolysis/coagulation variables

Carotenoids and eye health

Serum and adipose tissue concentrations of lutein were stronger associated with macular pigment (MP) density in men than in women in our observational study. MP density was not influenced by decreases in serum carotenoids after SPE consumption over 1 year (Table 1). A stronger relation between serum lutein and MP density in men, and a possible negative association between adipose tissue lutein and MP density in women have also been described by Johnson *et al.* (2000). They suggested that there could be more competition for the uptake of lutein between adipose tissue and the eye in women than in men (Johnson *et al.*, 2000). Hammond *et al.* (2002) suggested that obese subjects have a lower MP density due to a lower intake of carotenoids or to competition between body fat and retina in the uptake of lutein and zeaxanthin. Differences in body composition between men and women could also explain our findings.

The question however remains why significant associations have been found between serum lutein concentrations and MP density cross-sectionally whereas no significant effects on MP density have been observed after long-term lutein decrease. In the SPE study, serum lutein decreased by 20%, 11%, and 0.6% after consumption of 17, 10 and 7 g/d of SPE, respectively. According to the regression equation of the cross-sectional data, these decreases would be expected to be associated with changes in MP density of 4.8%, 2.5% and 0.7%, respectively. In all groups, including the control group MP density actually increased somewhat. Although the 17 g/d group did show a (non-significant) decrease of 4.8% relative to the control group, the 10 g/d and 7 g/d groups showed no tendency for a decrease comparable with the regression equations. The (non-significant) decreases relative to the control group were 1.4% and 2.7%, respectively. These results show that MP density is not simply a mathematical derivative of decreased concentrations of carotenoids in serum.

Recently, a dietary supplementation study with lutein/zeaxanthin-rich vegetables (Johnson *et al.*, 2000) showed a beneficial effect on MP density of ca. 18%. However, the increases in serum concentrations were 2-fold, much higher than the decreases of lutein we observed in the SPE study. The decreases in serum lutein in the SPE intervention study were probably too small to detect a significant effect on MP density. Whether a change of 1–5% is functionally relevant in relation to a change in disease risk remains unanswered. In this case, the difference between

significant effects and clinically relevant effects is important. The difference in MP density between eyes of AMD patients and eyes of control subjects is ca. 30% (Bone *et al.*, 2000). We speculate that an explanation for the difference between the cross-sectional and intervention findings could be that the eye has a sparing mechanism for the accumulation of carotenoids. As serum concentrations decrease, the capacity of the retina to accumulate carotenoids may increase. The mechanism of accumulation of carotenoids in the eye is not yet established. Recently, carotenoid-binding proteins have been discovered in the eye (Yemelyanov *et al.*, 2001). Mechanistic studies in eyes of animals and extracts from human eyes in which isomers and oxidation products of carotenoids are measured could give more insight into the metabolism of lutein and zeaxanthin in the macula and carotenoid uptake and stabilization.

In conclusion, in our study the associations of serum and adipose tissue with MP density are stronger in men than in women, probably because of a difference in body composition. Future research should focus on the role of body composition in the distribution of carotenoids in the body after an increased or decreased intake of carotenoids. Not only the bioavailability in serum, but also the functional bioavailability, i.e. the concentrations of lutein and zeaxanthin in these tissues has to be studied after carotenoid intake. In addition, the competition between adipose tissue and the eye in the uptake of lutein and zeaxanthin after intake of these carotenoids should be studied in subjects with different body fat distributions, subjects with high or low BMI or high or low body fat percentage. Supplementation studies have shown an accumulation of lutein and zeaxanthin in MP density when there is an increased supply of carotenoids. However, decreased concentrations as shown in our study did not have an influence on MP density, may-be due to a sparing mechanism in the eye. If there is a sparing mechanism, it is important to known how long this sparing mechanism will continue. Negative longer-term effects or minor changes of a decreased supply of lutein and zeaxanthin to MP density cannot be excluded by our study.

Carotenoids and skin health

Skin sensitivity to solar irradiation was not associated with serum carotenoid status in the cross-sectional study and was not influenced by a decrease in serum carotenoids (Table 1). Recently, a dietary supplementation study with lycopene-

rich tomato paste (Stahl *et al.*, 2001), showed a beneficial effect on the erythema reaction (–40%). However, the increases in serum concentrations were 2-fold, much higher than the decreases (24% for lycopene) we observed in the SPE study. The modifying effect of melanin on the association between serum carotenoids and minimal erythema dose (MED) suggests that the combination of high melanin concentrations and carotenoids is important, especially where maintenance of the carotenoid concentrations is vital to avoid deleterious effects of UV light, such as in the retina of the eye and the skin (Edge *et al.*, 2000).
In conclusion, supplementation studies show that carotenoids could influence the sensitivity to sunlight as measured by MED but our findings suggest that carotenoid decreases has no significant influence on MED. The mechanism behind a possible protective effect, probably by their antioxidant function, effect on gene regulation or interactions with melanin, could be studied by mechanistic studies in human skin cell-lines.

Fruit and vegetables, carotenoids and cardiovascular health

Although our fruit and vegetable study had limited statistical power, our findings do suggest that fruit and vegetables do not have a major impact on serum lipids and fibrinolysis/coagulation variables. Serum lipids were not influenced by a diet high in fruit and vegetables in another study (Obarzanek *et al.*, 2001). Fruit and vegetable consumption could influence blood pressure regulation and homocysteine concentrations. Indeed, Appel *et al.* (1997) and Svetsky *et al.* (1999) showed that a diet high in fruit and vegetables could decrease systolic blood pressure and diastolic blood pressure. Our findings on blood pressure were within the range of the reported blood pressure-lowering effects of high fruit and vegetable consumption in that earlier study. In accordance with our findings, Brouwer *et al.* (1999) observed a decrease in homocysteine after 4 weeks of citrus fruit and vegetable consumption. In contrast, Appel *et al.* (2000) did not show a significant difference in homocysteine between the control group and the fruit and vegetable group. The mean baseline homocysteine concentrations differed between the studies, with the highest baseline levels in our study followed by the studies of Brouwer *et al.* (1999) and Appel *et al.* (2000), probably in part explaining the differences in observed decreases of homocysteine.

Atherosclerosis is currently seen as an inflammatory disease (Ross, 1999). In our cross-sectional studies it was shown that carotenoids are related to inflammation markers and markers of endothelial function, such as C-reactive protein (CRP), leucocytes and soluble intercellular adhesion molecule (sICAM-1). A possible mechanism could be that carotenoids have anti-inflammatory effects. However, in our SPE study decreases in carotenoids had no effect on the inflammation markers. This could indicate that a third factor could influence both carotenoids and inflammation markers, for example, obesity. In studying the relation between fruit and vegetables, carotenoids and cardiovascular disease, inflammatory processes have to be taken into account (Kritchevsky *et al.*, 2000; Kritchevsky, 1999).

Validity of biomarkers

Macula pigment (MP) density as predictive marker for age-related macular degeneration

Whether a change in MP density reflects a change in risk for age-related macular degeneration (AMD) is not yet established. It is biologically plausible that MP density plays a role in the development of AMD. MP density can absorb blue light, which is harmful to the eye, or it can act as antioxidant to prevent oxidative damage to photoreceptors of the eye (Landrum and Bone, 2001). Furthermore, low MP density is associated to risk factors of AMD, such as iris color, gender and smoking. Bone *et al.* (2000) showed that the retina of donors with AMD had 30% lower MP density than the retina of control donors without AMD. They showed that those in the highest quartile of macular pigment had 82% lower risk for AMD than those in the lowest quartile. In contrast, Berendschot *et al.* (in press) showed that there was no difference in MP density between healthy subjects and patients with different stages of AMD. Differences between these studies in study material (subjects and donor eyes), techniques (Bone used HLPC while Berendschot used reflectance), and different disease stages makes it difficult to compare these results. It has been suggested that AMD is multifactorial. In addition to the possible protective role of carotenoids in MP, other components with antioxidant capacity could prevent the oxidative stress in the eye, such as vitamins C and E, and cofactors of antioxidant enzymes, like minerals. Other nutrients could also have an influence on AMD, such as fatty acids as important components of photoreceptors.

Other hypotheses are related to vascular damage in the eye. If MP density is part of the mainstream causal pathway relevant to the majority of disease cases, it could be a valid marker, despite other processes involved in the development of AMD.
Further research should be focused on the relation between MP density and AMD. Measurement of MP density in patients with AMD as compared to healthy controls could contribute to the biological plausibility of MP density as marker of AMD. In these studies a decrease of MP density in patients could be a cause or consequence of AMD. To study the predictive value of MP density on AMD, cohort studies or cohort-nested case-control studies could be used to evaluate the differences in MP density measured at the start of the cohort study and during follow-up in patients and controls. These studies are very expensive and time-consuming.
To validate the notion that a change in MP density reflects a change in AMD as a result of, for example, dietary modulation requires intervention studies with high-risk groups, for example patients with AMD in at least one eye. This could reduce the study period. By studying the effect of dietary modulation on MP density and the progression of AMD measured by photographs of the eye or visual acuity loss could elucidate the predictive value of MP density for the further development of AMD. Once the predictive value of MP density is established human intervention trials could be performed with MP density as endpoint.

Lens density as a predictive marker for cataract

Lens density increases with age, with a rapid acceleration after age 50. Lens density measured *in vivo* is associated with the accumulation of protein aggregates in the lens (Mota *et al.*, 1992). However, lens density varies among younger subjects with no discernible lens opacities (Hammond *et al.*, 1997). Observational studies show that lens density in subjects with cataract is higher than in age-matched subjects without cataract (Sample *et al.*, 1988). There are no longitudinal data available evaluating the predictive value of lens density early in life for cataract risk later in life.
Following subjects over time in cohort studies or cohort-nested case-control studies could elucidate the predictive value of lens density early in life for cataract risk later in life. Such studies could detect other factors that may be responsible for the difference in a common pattern of lens aging in most individuals and the accelerated pattern of lens aging in subjects who develop cataract.

To validate that a change in lens density reflects a change in cataract by dietary modulation, intervention studies are required with high-risk groups, for example diabetes patients, patients with cataract in at least one eye or patients who had previously one lens with cataract removed. The effect of the dietary intervention could be studied on lens density and the progression of cataract measured by opacity grades, cataract surgery or vision acuity loss.

Minimal erythema dose (MED) as a predictive marker for non-melanoma skin cancer

MED is a direct effect marker of personal sensitivity to sunburn. Is it necessary to validate MED as marker of non-melanoma skin cancer? The marker is used as a sensitivity marker, indicating that subjects with low MED values have to be careful with high sun exposure. There is persuasive evidence that sun exposure is a causal factor in the development of skin cancer. Observational studies have shown that the incidence rate of skin cancer is higher in fair-skinned, sun-sensitive subjects than in dark-skinned, less sun-sensitive subjects (Armstrong and Kricker, 2001). In observational studies sensitivity to sunlight is based on the comparison between ethnic groups of different skin color, color of unexposed skin, ability to tan and different skin types. In subjects with a skin that tans poorly, exposure to 0.3–0.5 standard MED (sMED; 1 sMED is the effective dose of UV irradiation capable of causing minimal erythema in an average skin type 2) may be sufficient to lead to erythema. In persons with a less sun-sensitive skin that has been adapted through regular exposure, 3–5 sMED leads to erythema, a difference by a factor of 10 (Report Health Council of the Netherlands, 1994).

Next to sun exposure, including the pattern as well as the amount of sun exposure, other factors are important in the development of skin cancer. Individual differences in repair mechanisms of DNA damage and immunosuppression and gene expression by UV-light could have an influence on the development of mutations and cancer cells. The question is whether the acute effect to UV (sensitivity to sunlight) is related to the chronic effect of UV (DNA damage, mutation induction and skin cancer). Young *et al.* (1998) showed that DNA damage spectra are comparable with erythema spectra after different doses of UV in human volunteers and induced repair of DNA damage can prevent erythema formation (Stege *et al.*, 2000). Heenen *et al.* (2001) showed a weak correlation

between erythema response and DNA damage after UV exposure because of the large intraindividual variation in DNA damage response after exposure to the same relative dose of MED.

The development of skin cancer is a multifactorial process. Increasing fundamental understanding of the factors in mechanistic studies that are of influence on the development of skin cancer after UV exposure will reveal the precise relationship between sensitivity to sunlight and the development of skin cancer.

However, the most important factor in the development of skin cancer is the sun exposure itself. When subjects with a low MED, reflect high sensitivity to UV-light, are not exposed to UV-light, they will not develop skin cancer. But they have the chance to develop skin cancer if they are exposed to UV-light.

A change in erythema as observed in dietary supplementation studies is not comparable with the use of a sunscreen with a high protection factor. Others suggest that dietary intake may provide basal skin protection against sunburn (Stahl, 2000). The question remains if this small change in sunburn sensitivity is related to a change in disease risk.

FMD, ICAM and homocysteine as predictive biomarkers for cardiovascular disease

Flow-mediated vasodilation is related to several risk factors of cardiovascular disease. Neunteufl *et al.* (2000) showed that cardiovascular events occurred more frequently in chest-pain patients with a FMD lower than 10% in comparison with chest-pain patients with a FMD higher than 10%. In another study, FMD of the coronary arteries was predictive of future coronary events (Schachinger *et al.*, 2000).

Concentrations of adhesion molecules, such as sICAM-1, are elevated in patients with ischemic heart disease (Morisaki *et al.*, 1997) and coronary heart disease (Hwang *et al.*, 1997) and could predict future death of cardiovascular disease in patients with coronary artery disease (Blankenberg *et al.*, 2001). In a study of Ridker *et al.* (1998) healthy subjects at baseline in the highest quartile of sICAM-1 (> 260 ng/mL) had 80% higher risk of myocardial infarction than subjects in the lowest quartile (< 193 ng/mL). However, Malik *et al.* (2001) reported that sICAM was only moderately associated with vascular disease after correction for classical risk factors.

As to homocysteine, it is not yet established if higher homocysteine concentrations are a cause or a consequence of cardiovascular disease, because retrospective epidemiological studies find much stronger associations between homocysteine concentrations and cardiovascular disease than do prospective studies. A 3–4 μmol/L reduction in homocysteine should lower vascular disease risk by one third (Eikelboom *et al.*, 1999). As shown in observational epidemiological studies folic acid is associated with risk of cardiovascular disease (Eichholzer *et al.*, 2001). Recently, it was shown that folic acid reduced homocysteine concentrations and new blockage of the treated artery in patients who had undergone angioplasty (Schnyder *et al.*, 2001). The question is if this is mediated via a homocysteine-lowering effect. Human placebo-controlled intervention studies are required to study the homocysteine-lowering effects by components other than B-vitamins on cardiovascular disease risk.

In conclusion, for all previously mentioned biomarkers of cardiovascular disease it is biologically plausible that they play a role in the development of cardiovascular disease. It is likely that changes in these biomarkers are related to changes in disease risk, but it is not yet established. For these variables, there is no clear cut-off point for disease. Every decrease in each variable could contribute to the reduced risk of cardiovascular disease. The pattern of different variables is important in risk estimation. Additional epidemiological prospective studies are necessary to evaluate the predictive values of these markers. In addition, in intervention trials with high-risk groups, for example in subjects who suffered from cardiac events, the effect of manipulation of these markers on the disease outcome can be studied. Interventions may alter one factor, but other factors could be adversely affected. Thus, studies with hard endpoints remain critical.

Conclusion

This thesis reports on a number of studies on serum carotenoid concentrations in relation to several biomarkers of eye, skin and cardiovascular health.

With regard to macular pigment as a marker of eye health, we did find cross-sectional associations with serum carotenoids, but these were influenced by gender and possibly by body composition. We did not observe clear adverse effects of SPE-induced serum carotenoid decreases over one year, but we cannot exclude minor changes. This does not add support to the hypothesis that changes in serum

carotenoids are associated with age-related macular degeneration. However, the relation of especially lutein and zeaxanthin with macula degeneration remains plausible, information on the biological relevance of minor changes in macula pigment is lacking, and we cannot exclude effects over a period of more than one year. Moreover, interpretation is hampered by our limited knowledge of the kinetics and metabolism of carotenoids. Competition between tissues for carotenoid absorption and possible 'sparing' of carotenoids by the macular tissue are hypotheses that deserve further study. Finally, the predictive value of MP density for AMD should be studied in follow-up studies.

With regard to cardiovascular disease, we did find several cross-sectional associations with serum carotenoids that confirm previously found inverse associations between carotenoids and chronic disease risk. However, our intervention studies with SPE or fruits and vegetables did not show effects of carotenoid changes in a normal physiological range on the biomarkers studied. This is in line with previous intervention studies with high doses of β-carotene that did not show a preventive effect. Our fruit and vegetable study did show an effect on plasma homocysteine and our results on blood pressure, with limited statistical power, are within the range of a previously reported blood pressure-lowering effects of high fruit and vegetable consumption. The latter two effects, however, are mechanistically unlikely to be mediated by serum carotenoids.

For MED as a marker of skin sensitivity to sunburn, we found neither clear cross-sectional associations with serum carotenoids nor effects in our 1-year SPE carotenoid reduction trial. Previous studies have shown effects of high dietary intake of carotenoids, but our studies indicate that associations between MED and serum carotenoids in the normal physiological range are non-significant.

Overall, our studies used biomarkers that are indicative of biological processes currently thought to be important in disease etiology, but the predictive value has not been established. Therefore, our studies are not conclusive for disease risk, but they seem to add proof to the hypothesis that serum carotenoid decreases or increases within normal physiological ranges in periods up to one year, have no or limited impact on biomarkers related to eye, skin and cardiovascular health. Future studies, specifically on the metabolism of carotenoids and follow-up studies on disease incidence, should clarify this matter further.

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Summary

Observational epidemiological studies have consistently shown that high consumption of carotenoid-rich fruit and vegetables is associated with a reduced risk of chronic diseases, such as cardiovascular, some types of cancer, cataract and macular degeneration. Although the preventive mechanisms are not fully understood, many authorities now recommend an intake of more than 400 g/d of fruit and vegetables and carotenoids have been frequently hypothesized to explain in part the beneficial effect of fruit and vegetables. Because of the potential beneficial effects of carotenoids, the introduction of products in which fat was replaced by non-digestible sucrose polyesters (SPE) was met with scientific concern, since SPE intake lowers plasma levels of carotenoids. Intervention trials showed that high dose β-carotene supplementation has no protective effect or even exerts an increased risk. Therefore, it seems advisable to study the effects of those carotenoids that are most abundant in the diet at nutritionally relevant levels on disease risk. Because intervention studies with hard end-points are time-consuming and costly, the use of biomarkers as predictors of the actual effect of diet on disease could be a promising approach.

The objective of this thesis was to evaluate the relation between serum carotenoid status in a normal physiological range and biomarkers of eye, skin and cardiovascular health in man. In an observational study the quantitative relations between serum carotenoid concentrations and biomarkers of eye (macular pigment and lens density), skin (minimal erythema dose) and cardiovascular health (markers of endothelial function and inflammation) were studied. The observational results were obtained from the baseline of an intervention study designed to evaluate the effect of long-term carotenoid decreases, as caused by SPE on markers of oxidative damage and functional markers of eye (macular pigment), skin (minimal erythema dose) and cardiovascular health (flow-mediated vasodilation). In the second intervention study the effect of a high but realistic consumption of fruit and vegetables was studied on serum carotenoids, vitamin concentrations and biomarkers of cardiovascular health (homocysteine, serum lipids, blood pressure, coagulation and fibrinolysis variables). In the introduction of this thesis background information on the health effects of carotenoids in relation

to eye, skin and cardiovascular health is given. Furthermore, several biomarkers of eye, skin and cardiovascular health are described.

Observational study

The 380 subjects in our study were recruited from the pool of volunteers of TNO Nutrition and Food Research and through advertizing in local and regional newspapers and on television. We aimed to select Dutch volunteers with high and low fruit and vegetable consumption according to a brief questionnaire in order to reach a wide range in carotenoid exposure. Respondents in the highest quintile of fruit and vegetable consumption and those in the lowest tertile were selected. The final study group comprised 178 men aged 42 ± 15 years and 201 women aged 41 ± 13 years. Of these volunteers, 33% and 27% were smokers, respectively.

Eye health

Macular pigment (MP), concentrated in the central area of the retina in the eye, contains the carotenoids lutein and zeaxanthin. A low MP density could be a risk factor for age-related macular degeneration (AMD). In our study, the quantitative relations between serum and adipose tissue concentrations of lutein and MP density were stronger in men than in women (Chapter 2). In men, an increment of 1 standard deviation (SD) in serum lutein was associated with 13% higher MP density. An increment of 1 SD in adipose tissue lutein was associated with 11% higher MP density in men. The weaker associations in women could be a consequence of more competition between uptake of lutein in the eye or adipose tissue.

No associations were found of serum carotenoids and vitamins with lens density, measured as a marker of cataract risk (Chapter 3). Macular pigment, as a marker of lutein and zeaxanthin in the eye, the only carotenoids that have been found in the human lens, was inversely associated with lens density. An increment of 1 SD in MP density was associated with 2.7% lower lens density. This suggests that lutein and zeaxanthin in the eye may retard aging of the lens. However, the uptake of lutein and zeaxanthin in the eye is still not clear.

Skin health

Sunburn is a well-known acute effect of excessive UV exposure. Low-dose or brief exposure to UV irradiation is tolerated by the skin without noticeable or clinically relevant changes. Only after reaching a certain threshold, an inflammatory response is induced, which is clinically visible as erythema, redness of the skin. A frequently used measure for the skin's sensitivity to UV light is the minimal erythema dose (MED). One MED is the minimum amount of energy required to induce visible erythema, which can be defined as a uniform, clearly demarcated or just perceptible redness at 16–24 h after UV exposure. Thus, a low MED indicates a high sensitivity to sunburn. Male subjects were found to be more sensitive to UV irradiation than female subjects (Chapter 4). Skin melanin content, the brown color of the skin, which was positively associated with iris color in both sexes and with hair color in men, was the main phenotypical determinant of sensitivity to UV-irradiation. No associations were found between serum carotenoids and MED in the total study group. However, associations between carotenoids concentrations and MED showed a positive trend in subjects with melanin values above the median and a negative trend in subjects below the median. We do not have a clear biological explanation for this modifying effect of melanin on the association between carotenoids and MED.

Cardiovascular health

The vascular endothelium is the primary site of dysfunction in atherosclerosis. Endothelial cell dysfunction may be related to an increase in cellular oxidative stress, a dysbalance between anti-oxidative and pro-oxidative factors. Carotenoids and vitamins could have an antioxidant-mediated tempering influence on endothelial function and inflammation, thereby reducing the risk of atherosclerosis. High-sensitive C-reactive protein (CRP), fibrinogen (Fbg) and leucocytes were measured as markers of inflammation. Soluble intercellular adhesion molecule-1 (sICAM-1) and flow-mediated vasodilation (FMD) were measured as markers of endothelial function. In the total study group, lutein and lycopene were inversely related to sICAM-1. β-Carotene was inversely related to leucocytes and CRP. Vitamin C was inversely related to CRP, whereas α-tocopherol was positively related to CRP. Zeaxanthin was inversely related to FMD. No significant associations were found between carotenoids, vitamins and fibrinogen. The inverse

relations between carotenoids, vitamin C and sICAM-1, CRP and leucocytes may help to explain the possibly protective effect of carotenoids and vitamin C on atherosclerosis through an influence on inflammatory processes and endothelial function (Chapter 5).

Intervention studies

SPE products lower concentrations of serum carotenoids in short-term studies. Experimental studies on the longer-term effects on health of decreased carotenoid concentrations are lacking. A 1-year randomized, double blind, placebo-controlled parallel trial was performed (Chapter 6). A total of 380 subjects were randomly assigned to the treatment with intakes of 0, 7, 10 or 17 g/d SPE. SPE was given in the form of spreads (daily consumption), chips (snackwise consumption), or both. Of the 380 subjects, 341 completed the study. After 1 year of intervention, serum lipid-adjusted carotenoids showed the largest decrease (13–33%) in the group consuming both SPE chips and SPE spread (17 g/d) compared with the control group. Consumption of SPE spread (10 g/d SPE) or SPE chips (7 g/d SPE) decreased carotenoid concentrations by 11–29% and 0.2–21%, respectively. In comparison with the control group, serum lipid-adjusted α-tocopherol decreased significantly by 6–8% (all $P < 0.001$) in all SPE groups. No negative effects were observed on biomarkers of oxidative damage to DNA (comet assay), proteins (carbonyls), lipids (8-epi-prostaglanins $F_{2\alpha}$) and skin (minimal erythema dose), and functional biomarkers of eye health (macular pigment density), cardiovascular health (flow-mediated vasodilation), and immune status (antibodies against hepatitis B vaccination) despite the decreased concentrations of carotenoids. These results suggest that substantial decreases in serum carotenoid concentrations do not imply an increased risk for chronic diseases. However, the relation between the change in biomarkers and the change in disease risk has to be established.

A 4-week single-blind placebo-controlled human intervention study with 48 subjects was performed to study the effect of a high but realistic intake of fruit and vegetables on serum carotenoids, vitamins and biomarkers of cardiovascular health (Chapters 7 and 8). Of the 48 subjects, 47 completed the study. In this study serum carotenoid concentrations increased by 22–128% in the group consuming 500 g/d fruit and vegetables + 200 mL fruit juice ('high' group) as compared to the group consuming 100 g/d fruit and vegetables ('low' group). Plasma homocysteine

concentrations decreased by 11% and folic acid increased by 15% in the 'high' group relative to the 'low' group. No effects were observed on serum lipids, blood pressure and markers of coagulation and fibrinolysis. Although this study did not have sufficient statistical power, our findings suggest that fruit and vegetables do not have a major impact on serum lipids and fibrinolysis/coagulation variables. Our results on blood-pressure are within the range of a previously reported blood pressure lowering effects of high fruit and vegetable consumption. The effects on homocysteine and blood pressure however, are mechanistically unlikely to be mediated by serum carotenoids.

In Chapter 9, results described in this thesis, studying the relation between serum carotenoids in a normal physiological range and biomarkers are summarized and discussed. Furthermore, the predictive value of these biomarkers is discussed.

Overall, our studies used biomarkers that are indicative of biological processes currently thought to be important in disease etiology. But the predictive value has not been established. Therefore, our studies cannot be conclusive for disease risk, but they seem to add proof to the hypothesis that decreases or increases in serum carotenoid concentrations within normal physiological ranges in periods up to one year have no or limited impact on biomarkers related to eye, skin and cardiovascular health. Future studies, specifically on the metabolism of carotenoids and follow-up studies on disease incidence, should clarify this matter further.

Samenvatting

Observationele epidemiologische studies hebben aangetoond dat een hoge consumptie van groente en fruit, welke rijk zijn aan carotenoïden, geassocieerd is met een lager risico op chronische ziekten, zoals hart- en vaatziekten, verschillende vormen van kanker en de oogziekten staar en macula degeneratie. De preventieve mechanismen zijn nog niet helemaal bekend, maar de consumptie van meer dan 400 gram groente en fruit per dag wordt aanbevolen. De hypothese is dat het preventieve effect van groente en fruit voor een gedeelte verklaard kan worden door de aanwezigheid van carotenoïden in groente en fruit. Produkten met vetvervangers, zoals bijvoorbeeld sucrose polyesters (SPE) verlagen carotenoïden in het bloed. De introductie van deze producten op de markt werd met bezorgheid ontvangen in de wetenschappelijke wereld vanwege de daling in carotenoïden niveaus na consumptie van SPE. Interventie studies die het effect onderzochten van hoge doseringen β-caroteen laten geen preventieve effecten zien op ziekterisico. In enkele gevallen werd zelfs een verhoogd risico op ziekte aangetoond. Het is aan te raden om de effecten te bestuderen van een normale inname van carotenoïden op het ziekterisico. Interventie studies met harde eindpunten, zoals bijvoorbeeld het ontstaan van kanker, zijn kostbaar en tijdrovend. Het gebruik van biomarkers kan hierin uitkomst bieden. Biomarkers kunnen dienen als voorspellers van het effect van de voeding op de gezondheid of het uiteindelijk ontstaan van ziekte.
De doelstelling van dit proefschrift was om de relatie te bestuderen tussen normale fysiologische concentraties van carotenoïden in het bloed en biomarkers gerelateerd aan de gezondheid van het oog, huid en hart en bloedvaten in de mens. In een observationele studie werden de kwantitatieve relaties bestudeerd tussen carotenoïden in het serum en biomarkers van gezondheid van het oog (macula pigment dichtheid en lensdichtheid), huid (minimale erythema dosis), en hart en bloedvaten (markers van endotheel functie en ontsteking). Hiervoor werd gebruik gemaakt van de baseline gegevens van een interventie studie. In deze studie werd het effect bestudeerd van een langdurige daling van carotenoïden in het serum, als gevolg van SPE consumptie, op biomarkers van oxidatieve schade en functionele markers van de gezondheid van het oog (macula pigment), huid (minimale erythema dose) en hart en vaten (flow-mediated vasodilation). In een tweede interventie studie werd het effect van een hoge maar realistische inname van

groente en fruit bestudeerd op concentraties van carotenoïden en vitamines in het bloed en biomarkers gerelateerd aan de gezondheid van hart en bloedvaten (homocysteine, serum lipiden, bloeddruk, coagulatie en fibrinolyse variabelen).
In de introductie van dit proefschrift wordt achtergrond informatie gegeven over de gezondheidseffecten van carotenoïden in relatie tot het oog, huid en hart en bloedvaten. Verder worden er verschillende biomarkers die gerelateerd zijn aan de gezondheid van het oog, huid en hart en bloedvaten besproken.

Observationele studie
In onze studie werden 380 proefpersonen geworven via het proefpersonen bestand van TNO Voeding en via advertenties in lokale en regionale kranten en op televisie. We probeerden om proefpersonen met een hoge en lage dagelijkse groente en fruit inname te selecteren met behulp van een korte vragenlijst. Daardoor zou het bereik in de carotenoïden concentraties in het bloed breder zijn.
Van de proefpersonen die de vragenlijst retourneerden, selecteerden we de mensen in het hoogste kwintiel en het laagste tertiel van de dagelijkse groente en fruit inname. De uiteindelijke groep bestond uit 178 mannen en 201 vrouwen met een gemiddelde leeftijd van respectievelijk 42 ± 15 en 41 ± 13 jaar. Deze groepen bestonden voor 33% en 27% uit rokers.

Gezondheid van het oog
Macula pigment (MP) is geconcentreerd in het centrale gedeelte van het netvlies in het oog. Het bestaat uit de carotenoïden luteïne en zeaxanthine. Een lage MP dichtheid kan een risicofactor zijn voor het optreden van de ouderdoms oogziekte macula degeneratie. In onze studie waren de kwantitieve relaties tussen concentraties van luteïne in het serum en vetweefsel en macula pigment in mannen sterker dan in vrouwen (hoofdstuk 2). Een stijging van 1 standaard deviatie (SD) in serum luteïne was geassocieerd met een stijging van 13% in MP dichtheid. Een stijging van 1 SD in luteïne in vetweefsel was geassocieerd met een stijging van 11% in MP dichtheid. Het vinden van zwakkere associaties in vrouwen zou een gevolg kunnen zijn van een grotere competitie in de opname van luteïne in het oog of in het vetweefsel. Geen associaties werden gevonden tussen carotenoïden en vitamines in het bloed en lens dichtheid, een marker voor het optreden van staar (hoofdstuk 3). Macula pigment als marker voor luteïne en zeaxanthine in het oog,

de enige carotenoïden die ook in de ooglens aanwezig zijn, was negatief geassocieerd met lens dichtheid. Dit suggereert dat luteïne en zeaxanthine in het oog de veroudering van de lens tegen kunnen gaan. Het opname mechanisme van luteïne en zeaxanthine in het oog is echter nog niet bekend.

Gezondheid van de huid

Verbranding van de huid is een bekend acuut effect van blootstelling aan ultaviolet (UV) licht. Blootstelling aan een lage dosis UV of een korte blootstelling wordt getolereerd door de huid. Alleen bij het bereiken van een drempel wordt een ontstekings respons geïnduceerd, waardoor de huid rood kleurt (erytheem). Een bekende maat voor het meten van de gevoeligheid van de huid voor UV licht is de minimale erythema dosis (MED). Eén MED komt overeen met de minimale hoeveelheid energie, die nodig is voor het induceren van net visueel zichtbare roodheid van de huid, 16 tot 24 uur na blootstelling. Dus een lage MED komt overeen met een hoge gevoeligheid voor zonlicht. Mannen waren gevoeliger voor zonlicht dan vrouwen (hoofdstuk 4). De concentratie van melanine in de huid, waardoor de huid bruin kleurt, was geassocieerd met de iriskleur in mannen en vrouwen en haarkleur in mannen. De melanine concentratie was de belangrijkste fenotypische determinant voor de gevoeligheid voor zonlicht. We vonden geen associaties tussen carotenoïden en MED in de totale studie groep. Echter, de associaties tussen carotenoïden en MED laten een positieve trend zien in mensen met een donkere huid (hoog melanine) en een negatieve trend in mensen met een lichte huid (laag melanine). Voor dit verstorende effect van melanine op deze associaties hebben we geen duidelijke biologische verklaring.

Gezondheid van hart en bloedvaten

Bij atherosclerose (aderverkalking) is het evenwicht in het endotheel van de vaatwand verstoord. Deze verstoring zou gerelateerd kunnen zijn aan oxidatieve stress, een verstoorde balans tussen anti-oxidatieve en pro-oxidatieve factoren. Carotenoïden en vitamines zouden vanwege de antioxidant capaciteit een invloed kunnen hebben op endotheelfunctie en ontstekingsprocessen, waardoor het risico op atherosclerose verlaagd kan worden. C-reactief eiwit (CRP), fibrinogeen (Fbg) en witte bloedcellen werden gemeten als markers van ontsteking. Intercellulair adhesie-eiwit (sICAM-1) en flow-mediated vasodilation (FMD; vaatverwijding

door toegenomen bloeddoorstroming) werden gemeten als markers van endotheel functie. In de totale studie groep, waren luteïne en lycopene negatief geassocieerd met sICAM-1. β-Carotene was negatief geassocieerd met CRP en leukocyten. Vitamine C was negatief geassocieerd met CRP en vitamine E was positief geassocieerd met CRP. Zeaxanthine was inverse geassocieerd met endotheel functie, gemeten met FMD. Er werden geen associaties gevonden tussen fibrinogeen en carotenoïden en vitamines. Deze inverse associaties tussen carotenoïden, vitamin C en sICAM-1, CRP en witte bloedcellen dragen bij aan de mogelijke verklaring dat carotenoïden en vitamine C een beschermend effect hebben op het risico voor atherosclerose als gevolg van een invloed op ontstekingsprocessen en endotheelfunctie.

Interventie studies

Resultaten van korte termijn studies laten zien dat produkten met SPE de concentraties van carotenoïden in het bloed verlagen. Studies waarin de lange termijn effecten van deze verlaagde carotenoïden concentraties bestudeerd worden, zijn nog niet uitgevoerd. Daarom hebben wij een 1-jaar durende, gerandomiseerde, dubbel-blinde, placebo-gecontroleerde studie uitgevoerd. 380 mensen werden verdeeld over 4 behandelingen met een inname van 0, 7, 10 of 17 gram SPE per dag (hoofdstuk 6). Dit werd gegeven in de vorm van smeersel (dagelijkse consumptie), chips (snack consumptie) of beide. Van de 380 mensen hebben 341 mensen de studie afgemaakt. Na één jaar waren de carotenoïden concentraties (aangepast aan lipiden concentraties in het bloed; gestandaardiseerd) gedaald met 13–33% in de groep die zowel chips als smeersel met SPE consumeerde (17 gram/dag) in vergelijking met de controle groep (0 gram/dag SPE). In de groepen die alleen smeersel met SPE (10 gram/dag SPE) of chips met SPE (7 gram/dag SPE) consumeerden, daalden de gestandaardiseerde carotenoïden concentraties respectievelijk met 11 tot 29% en 0,2 tot 21%.

In vergelijking met de controle groep, daalden de gestandaardiseerde α-tocopherol concentraties met 6–8% ($P < 0.001$) in alle SPE groepen. Ondanks de sterke dalingen in carotenoïden concentraties, werden er geen negatieve effecten gevonden op biomarkers voor oxidatieve schade aan DNA, het erfelijk materiaal van de mens (comet assay), vetten (8-epi-prostaglandines $F_{2\alpha}$), eiwitten (carbonyls) en de huid (minimal erythema dosis), en functionele biomarkers voor de

gezondheid van het oog (macula pigment dichtheid), hart en vaten (FMD) en immuun status (geproduceerde antilichamen na een hepatitis B vaccinatie). Deze studie suggereert dat een behoorlijke carotenoïden daling niet geassocieerd is met een verhoogd risico op chronische ziekten. Echter, de relatie tussen een verandering in deze biomarkers en een verandering in het risico op ziekte moet nog worden vastgesteld.

In een tweede interventiestudie (enkel-blind, placebo-gecontroleerd) van 4 weken met 48 mensen werd het effect bestudeerd van een hoge maar realistische inname van groente en fruit op carotenoïden, vitamines in het bloed en biomarkers van hart- en vaatgezondheid (hoofdstuk 7 en 8). In deze studie stegen de carotenoïden concentraties met 22–128% in de groep waarin de mensen 500 gram groente en fruit en 200 ml vruchtensap (hoge groep) per dag consumeerden in vergelijking met de groep waarin de mensen 100 gram groente en fruit (lage groep) per dag consumeerden. Homocysteïne concentraties daalden met 11% en foliumzuur concentraties stegen met 15% in de hoge groep in vergelijking met de lage groep. Geen effecten werden gevonden op bloedlipiden, bloeddruk en markers van coagulatie en fibrinolyse. Onze resultaten voor bloeddruk vallen binnen het bereik van de resultaten van een ander onderzoek, waarin werd aangetoond dat na een hoge groente en fruitconsumptie de bloeddruk kan dalen. De effecten op homocysteïne en bloeddruk na groente en fruit consumptie zijn waarschijnlijk niet gerelateerd aan de stijgende carotenoïden concentraties.

In hoofdstuk 9 worden de resultaten die beschreven zijn in dit proefschrift samengevat en bediscussieerd. Verder wordt de voorspellende waarde voor ziekte en gezondheid van de verschillende biomarkers bediscussieerd. In onze studies maakten we gebruik van biomarkers die indicatief zijn voor biologische processen, die mogelijk ten grondslag liggen aan de ontwikkeling van ziekten. Van deze biomarkers is de voorspellende waarde voor het ontstaan van ziekte alleen nog niet vastgesteld. Daarom kunnen we uit onze studies geen conclusies trekken wat betreft ziekterisico. Echter, deze resultaten dragen bij aan de hypothese dat stijgingen en dalingen van serum carotenoïden binnen een normaal fysiologisch bereik, geen effect hebben op biomarkers gerelateerd aan de gezondheid van het oog, huid en hart en bloedvaten. Verdere studies die het metabolisme van carotenoïden bestuderen en de incidentie van ziekte zouden kunnen bijdragen aan dit onderwerp.

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About the author

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