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Jouni Karppi

Measurement of Carotenoids and Their Role in Lipid Oxidation and Cancer

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UNIVERSITY OF
EASTERN FINLAND

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ABSTRACT

Carotenoids are colourful compounds, present in fruits and vegetables, synthesised by plants and micro-organisms. About 10% of these are important dietary precursors of vitamin A. Carotenoids act as antioxidants and possibly decrease in-vivo lipid oxidation. Lipid oxidation is known to be a risk factor for atherosclerosis. Carotenoids are thought to be responsible for the beneficial properties of fruits and vegetables in preventing human diseases, including cardiovascular diseases and cancer. In recent years, the antioxidant properties of carotenoids have become a major focus of research. The aim of this thesis was to develop a high performance liquid chromatographic (HPLC) method for determination of carotenoids from blood plasma and to study the role of carotenoids in lipid oxidation and cancer.

We developed and validated an HPLC method for analysis of carotenoids (lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene) that appears to be simple, quick and repeatable. Serum concentrations of carotenoids, except for lycopene, tended to increase in men and women as they became older, indicating an increase in the consumption of fruits and vegetables from the late 1980s to the beginning of the 2000s. The decrease in lycopene concentrations found in both sexes during follow-up years suggests that elderly people may not consume as many tomatoes and tomato products as do young people.

We investigated the effects of astaxanthin supplementation (8 mg/d) on lipid oxidation in healthy men and its safety as a supplement. When supplemented as capsules, astaxanthin was efficiently absorbed from the intestine into the blood circulation and was well tolerated. An almost significant decrease was found in 15-hydroxy fatty acid concentration after astaxanthin supplementation for three months. The serum low density lipoprotein (LDL) content of conjugated dienes is an in vivo lipid oxidation marker. We observed that in addition to gender, lycopene, lutein and β-carotene were the most powerful determinants for serum LDL conjugated dienes in Eastern Finnish men and women. A diet rich in vegetables and carotenoids can decrease in vivo LDL oxidation and thus slow down atherogenesis. We also studied the association between the serum concentration of lycopene and the risk of cancer. Men with serum lycopene concentrations higher than 0.19 μmol/l had a 45% lower risk for total cancer than did men with lycopene under 0.08 μmol/l. However, lycopene was not associated with prostate cancer in this population.

In conclusion, serum/plasma carotenoids may decrease lipid oxidation in vivo. In addition, high serum concentrations of lycopene may decrease the risk of cancer in middle-aged Finnish men.

National Library of Medicine Classification: QV 325, QU 110, QU 85, QZ 200

Medical Subject Headings (MeSH): Antioxidants; Carotenoids/blood; Cholesterol; chromatography, High Pressure Liquid; Lipoproteins, LDL; Lipids/blood; Lipid Peroxidation; Neoplasms

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TIIVISTELMÄ

Karotenoidit ovat kasvien ja pieneliöiden (levät, bakteerit, sienet) tuottamia värillisiä yhdisteitä, jotka antavat värin niitä sisältäville hedelmille ja vihanneksille. Noin 10% kaikista karotenoideista on A-vitamiinin esiasteita. Karotenoidit toimivat elimistössä antioksidantteina ja vähentävät mahdollisesti rasvojen liiallista hapettumista, joka on ateroskleroosin riskitekijä. Karotenoidipitoisten kasvisten runsaan saannin on todettu mm. vähentävän riskiä sairastua sydän- ja verisonitauteihin, syöpätauteihin ja muihin kroonisiin sairauksiin. Tämän väitöskirjatyön tarkoituksena oli kehittää nestekromatografinen menetelmä karotetoidien määrittämiseksi veriplasmasta sekä tutkia karotenoidien merkitystä elimistön rasvojen hapettumisessa ja syöpätaudeissa.

Kehitimme ja validoimme karotenoideille (luteiini, zeaksantiini, β-kryptoksantiini, lykopeeni, α- ja β-karoteeni) nestekromatografiamenetelmän, joka osoittautui helpoksi, nopeaksi ja toistettavaksi. Itäsuomalaisessa väestössä karotenoidien pitoisuudet nousivat naisilla ja miehillä heidän ikääntyessään, joka osoittaa hedelmien ja vihannesten käytön lisääntyneen 1980-luvulta 2000-luvulle. Sitä vastoin seurannan perusteella näyttäisi, että iän myötä itäsuomalaiset syövät vähemmän tomaatteja ja tomaattipohjaisia elintarvikkeita kuin nuoremmat, koska seerumin lykopeenipitoisuus väheni tutkittavien ikääntyessä.

Selvitimme astaksantiinilisän (8 mg/vrk) vaikutusta ihmisen elimistön rasvojen hapettumiseen, astaksantiinin imeytymiseen, plasmapitoisuuksiin ja arvioimme astaksantiinin turvallisuutta ravintolisänä. Astaksantiini imeytyi hyvin kapseleista verenkiertoon ja oli hyvin siedetty. 15-hydroksirasvahapon pitoisuudessa havaittiin lähes merkitsevä väheneminen kolmen kuukauden astaksantiinilisän käytön jälkeen. Seerumin LDL:n konjugoituneiden dieenien määrää mittaamalla saadaan tietoa LDL:n in vivo hapettumisesta. Havaitsimme, että sukupuolen lisäksi plasman lykopeeni, luteiini ja β-karoteeni ovat tärkeimmät seerumin LDL:n konjugoituneiden dieenien määrään vaikuttavat tekijät itäsuomalaisten miesten ja naisten aineistossa. Kasvisravinnosta saatavat karotenoidit saattavat vähentää hapetusreaktioiden aiheuttamia muutoksia LDL:ssä ja hidastaa aterogeneesiä. Tutkimme myös seerumin lykopeenin ja syöpäriskin välistä yhteyttä. Havaitsimme kokonaissyöpäriskin olevan 45% pienempi miehillä, joiden seerumin lykopeenipitoisuus oli yli 0.19 μmol/l kuin miehillä, joilla se oli alle 0.08 umol/l. Pelkästään eturauhassyöpään ei lykopeenilla ollut tässä aineistossa yhteyttä.

Työssä havaittiin, että korkeat karotenoidipitoisuudet seerumissa/plasmassa saattavat vähentää elimistön rasvojen hapettumista. Lisäksi korkea lykopeenipitoisuus seerumissa voi vähentää kokonaissyöpäriskiä keski-ikäisillä itäsuomalaisilla miehillä.

Yleinen suomalainen asiasanasto (YSA): antioksidantit; karotenoidit; lykopeeni; lipidit−veri; lipidit−hapettuminen; nestekromatografia; LDL-kolesteroli; syöpätaudit

To my family with love

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Kuopio, February 2011

Jouni Karppi

List of original publications

This dissertation is based on the following articles which are referred to in the text by Roman numerals:

I Karppi J, Nurmi T, Olmedilla-Alonso B, Granado-Lorencio F, Nyyssönen K. Simultaneous measurement of retinol, α-tocopherol and six carotenoids in human plasma by using an isocratic reversed-phase HPLC method. J Chromatogr B Analyt Technol Biomed Life Sci 2008;867:226-32.

II Karppi J, Rissanen TH, Nyyssönen K, Kaikkonen J, Olsson AG, Voutilainen S, Salonen JT. Effects of astaxanthin supplementation on lipid peroxidation. Int J Vitam Nutr Res 2007;1:3-11.

III Karppi J, Nurmi T, Kurl S, Rissanen TH, Nyyssönen K. Lycopene, lutein and β-carotene as determinants of LDL conjugated dienes in serum. Atherosclerosis 2010;209(2):565-72.

IV Karppi J, Kurl S, Nurmi T, Rissanen TH, Pukkala E, Nyyssönen K. Serum lycopene and the risk of cancer: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study Ann Epidemiol 2009;19(7):512-8.

Contents

TABLES

FIGURES

ABBREVIATIONS

$^1O_2$	Singlet molecular oxygen
AAPH	2,2-azobis(2-amidinopropane) dihydrochloride
AMI	Acute myocardial infarction
ANOVA	Analysis of variance
ApoB	Apolipoprotein B
Apo-CAR	Apo-carotenoids
ASTA	Astaxanthin supplementation study
ATBC	Alpha-tocopherol, beta-carotene cancer prevention study
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BMI	Body mass index
BPH	Benign prostate hyperplasia
C_8	Octylsilyl
C_{18}	Octadecylsilyl
C_{30}	Triacontanylsilyl
CAD	Coronary artery disease
CAR	Carotenoid
$CAR^{\bullet}$	Carotenoid radical
CARET	The Beta-Carotene and Retinol Efficacy Trial
CEC	Capillary electrochromatography
CHD	Coronary heart disease
CI	Confidence interval
CRP	C-reactive protein
CV	Coefficient of variance
CVD	Cardiovascular diseases
CYP1A1	Cytochrome p450 enzyme 1A1
DAD	Diode array detector
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
ED	Electrochemical detector
EDTA	Etylene diamine tetra-acetic acid
EPIC	The European Prospective Investigation into Cancer and Nutrition
FDA	The U.S. Food and Drug Administration
FOX	Ferrous oxidation
γ-GT	Gamma-glytamyl transferase
GC	Gas chromatography
H^+	Proton
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HOCl	Hypochlorous acid
HPFS	Health Professionals Follow-Up Study
HPLC	High performance liquid chromatography

IHD	Ischaemic heart disease
IU	International unit
KIHD	Kuopio Ischaemic Heart Disease Risk Factor Study
Lag time	Time to maximal oxidation rate
LDL	Low density lipoprotein
LOD	Limit of detection
MeO-AMVN	4-methoxy-2,4-dimethylvaleronitrile
MDA	Malondialdehyde
MSKCC	Memorial Sloan-Kettering Cancer Centre
N	Number
NADH	Nicotinamide adenine dehydrogenase
ND	Not determined
NHS	Nurse's Healthy Study
NIST	National Institute of Standards and Technology
NO	Nitric oxide
$NO^{\bullet}_2$	Nitrogen dioxide radical
NO_2	Nitrogen dioxide
NR	Not reported
$O_2^{-\bullet}$	Superoxide radical
O_3	Ozone
OW	Optothermal window
Ox-LDL	Oxidized low density lipoprotein
PON	Paraoxonase
PSA	Prostate specific antigen
PUFA	Polyunsaturated fatty acids
QC	Quality control
r	Correlation coefficient
$R^{\bullet}$	Lipid radical
RAL	Retinal
RDA	Recommended diatary allowances
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RR	Relative risk
SD	Standard deviation
SOD	Super oxide dismutase
SPE	Solid phase extraction
SUVIMAX	The Supplementation en Vitamines et Mineraux Antioxydants
t(1/2)	Elimination half-life
TBARS	Thiobarbituric acid reactive substances
TG	Triglycerides
TLC	Thin layer chromatography
TOH	Tocopherol
UV	Ultraviolet

VITAL	The VITamins And Lifestyle study
VLDL	Very low density lipoprotein
V_{max}	Maximim reaction velocity

1 Introduction

Reactive oxygen species (ROS) (i.e., free radicals) form through normal aerobic metabolism. Life-style (smoking, alcohol) and diet produce free radicals that can damage biological macromolecules, such as proteins, DNA, cholesterol and polyunsaturated fatty acids of LDL (Tapiero et al. 2004). This oxidative stress has been suggested as leading to an increased risk of chronic degenerative diseases, such as cardiovascular diseases (Witztum 1994) and cancers (Ames et al. 1995). Dietary antioxidants including carotenoids from fruits and vegetables have been shown to be effective compounds for preventing the risk of chronic diseases by reducing oxidative stress (Jackson et al. 2008).

Carotenoids are widespread in nature and are prevalently found in plants, animals, and microorganisms. Carotenoid-like pigments are responsible for the yellow, orange, and red colors of various fruits, vegetables, flowers, birds, fish, and crustaceans (Jackson et al. 2008). They are also used as natural colouring agents in the food industry. Lycopene, α-carotene, β-carotene, lutein, zeaxanthin and β-cryptoxanthin are major carotenoids found in Western diet (Krinsky & Johnson 2005).

Carotenoids have various biological effects on human health. The antioxidant activity of carotenoids has been suggested as having a significant beneficial effect on health (Rao & Rao 2007). Recent studies have shown that carotenoids may contribute to other mechanisms, including gap junction communication, cell growth regulation, modulating gene expression, immune response and as modulators of drug metabolizing enzymes (Paiva & Russell 1999; Astrog 1997; Bertram 1999; Jewell & O'Brien 1999).

Many previous epidemiological and intervention studies support a role for carotenoids in the prevention of lipid oxidation in vivo (Kioskias & Gordon 2003; Iwamoto et al. 2000; Visioli et al. 2003; Chopra et al. 2000), though this effect has not been observed in all studies (Hininger et al. 2001; Carroll et al. 2000; Freese et al. 2002). A number of epidemiological studies have shown an inverse association between carotenoid intake/plasma concentrations and cancers (Zhang et al. 2007; Lee et al. 2009a; Jenab et al. 2006; Jiang et al. 2005), but the results have been inconsistent (Peters et al. 2007; Albanes et al. 1995; Männisto et al. 2007; Gallicchio et al. 2008).

The aim of this work was to develop a rapid, simple method for analyzing carotenoids from blood plasma, and to study the role of carotenoids in lipid oxidation in elderly men and women and the risk of cancer in middle-aged men living in Eastern Finland.

2 Review of the literature

2.1 CAROTENOIDS

Carotenoids are a group of colourful compounds that are synthesised by plants and micro-organisms, but not by animals (Rao & Rao 2007). In 1831, Wachenroder crystallized a hydrocarbon pigment from carrot roots that he called "carotene" (Wachenroder 1831). Soon thereafter, Berzelius extracted the more polar yellow pigments from autumn leaves and called them "xanthophylls" (Berzelius 1837). Tswett separated numerous pigments of this class chromatographically and began to call the whole group "carotenoids" (Tswett 1911). Today, more than 750 carotenoids have been isolated from natural sources, of which about 50 are present in the human diet and about 20 have been identified in human blood and tissues (Rao & Rao 2007). The most common carotenoids in the human body are lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene (close to 90% of all carotenoids) (Rao & Rao 2007). Astaxanthin is synthesised by plants and algae (e.g. *haematococcus pluvialis*) and is distributed mainly in aquatic animals including salmon, trout, red seabream, shrimp, lobster, and fish eggs (Guerin et al. 2003).

2.1.1 Chemistry

The structure of carotenoids is based on a C_{40} isoprenoid skeleton that may be acyclic or have one or both ends modified into rings. All C_{40} carotenoids are derived from the acyclic tetraterpene, lycopene. Lycopene is biosynthesized from a total of eight C_5 isoprene units. Initially, four C_5 units combine to produce the C_{20} intermediate geranylgeranyl diphosphate, and two C_{20} precursors combine in a head-to-head fashion to form the C_{40} intermediate phytoene, the more saturated precursor of lycopene. Other carotenoids are synthesized from lycopene by modifications, such as cyclizations, oxidative functionalizations, rearrangements, and oxidative degradations (Jackson et al. 2008). The molecular structures of common carotenoids and astaxanthin are presented in Figure 1.

Carotenoids are divided into hydrocarbon carotenoids and xanthophylls (Jackson et al. 2008). Lycopene, α-carotene and β-carotene belong to the class of hydrocarbon carotenoids. They contain 11 conjugated double bonds and two non-conjugated double bonds. Xanthophylls such as astaxanthin, lutein, zeaxanthin, β-cryptoxanthin also have 11 conjugated double bonds and at least one hydroxyl group, due to which they are more polar than hydrocarbon carotenoids. Approximately 50 of the known carotenoids are precursors of vitamin A (Krinsky & Johnson 2005). Owing to its two unsubstituted β-ionone rings at the ends of the isoprenoid chain, β-carotene is the carotenoid with the highest pro-vitamin A activity, while other carotenoids such as α-carotene and β-cryptoxanthin have lower activities (Yonekura & Nagao 2007).

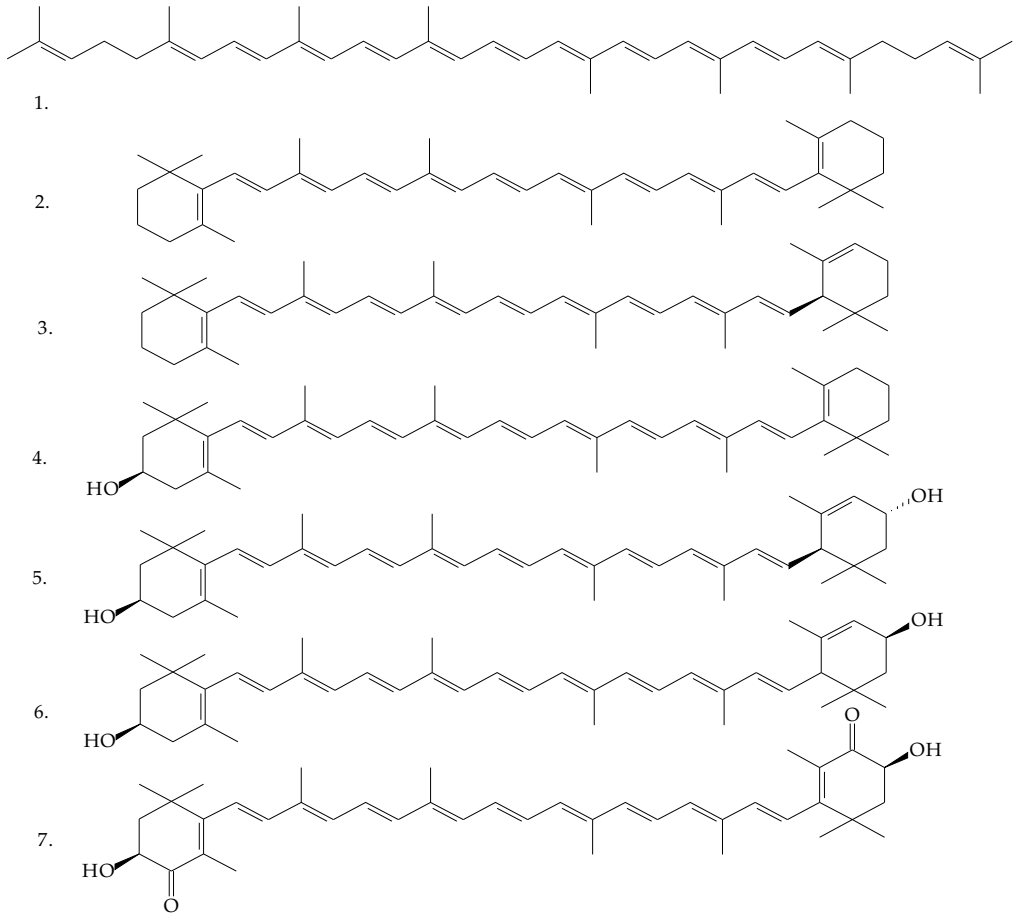

Figure 1. Molecular structures of common carotenoids and xanthophylls. Carotenoids: 1. Lycopene, 2. β-Carotene, 3. α-Carotene. Xanthophylls: 4. β-Cryptoxanthin, 5. Lutein, 6. Zeaxanthin, 7. Astaxanthin.

Carotenoids have highly conjugated double-bond chains and therefore they absorb light at the wavelength range of 400 to 500 nm. Absorption spectra of carotenoids contain three peaks, or two peaks and a shoulder (De Leenher et al. 2000).

Natural carotenoids occur mainly in their thermodynamically more stable all-trans configuration, while the cis isomers are only present in minor amounts and have been demonstrated to be formed as a consequence of food processing, such as heating and illumination (Aman et al. 2005). Cis-trans isomerization may occur at any double bond of the carotenoid polyene chain, leading to a large number of mono-and poly-cis isomers (Britton 1995). It has been observed that in human serum and tissues, more than 50% of the lycopene and ~5% of the β-carotene exists

in the cis conformation (Stahl et al. 1992). They are unstable in the presence of light, oxygen and heat. Most of carotenoids are singlet oxygen quenchers (Di Mascio et al. 1990), of which lycopene is the most efficient because it contains the highest number of double bonds (Di Mascio et al. 1989).

Astaxanthin contains hydroxyl and keto endings on each ionone ring that explain some unique properties; such a translocation of the terminal rings of astaxanthin should be advantageous for scavenging the lipid peroxyl radicals in the membrane and the reactive oxygen species at the membrane surface. Indeed, it has been suggested that astaxanthin may scavenge radicals inside the membrane both by the conjugated polyene chain and the terminal ring moiety (Goto et al. 2001) (Figure 2).

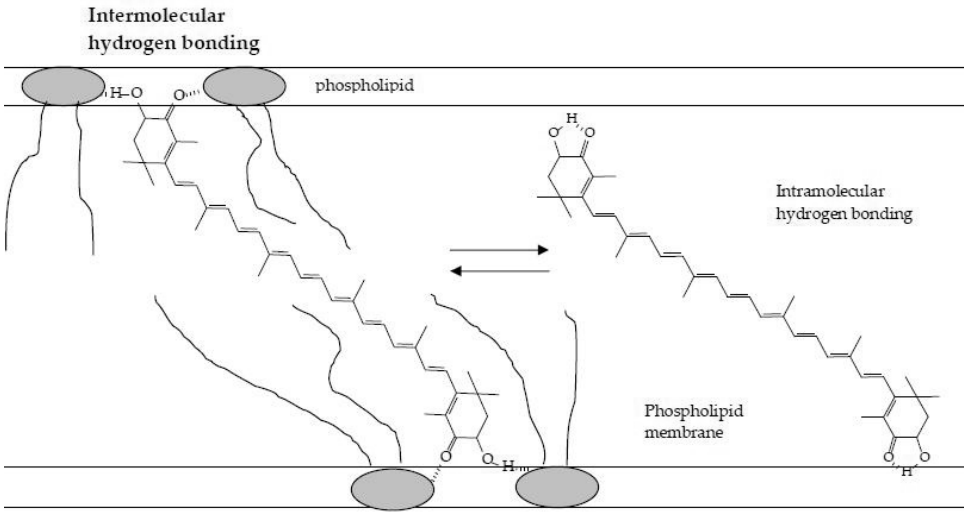

Figure 2. Schematic representation of the possible locations of astaxanthin molecules having inter- and intramolecular hydrogen bonds in the phospholipid membrane.

2.1.2 Bioavailability and metabolism

The release of carotenoids from a food matrix is an important initial step in its absorption. Carotenoids are absorbed better from heat processed plant foods than from unprocessed sources, with the the absorption being increased by dietary fat (Yonekura & Nagao 2007; Bohm & Bitsch 1999; Stahl & Sies 1992). For instance, the amount of β-carotene absorbed from cooked carrots has been found to be 65% and from raw carrots 41% (Livny et al. 2003). Absorption of carotenoids in the gastrointestinal tract is <50%, with the rest being excreted with the feces (Erdman et al. 1993b). After dissociation of protein-carotenoid complexes, carotenoids are emulsified into small lipid droplets in the stomach and transferred into mixed micelles (composed of bile salts, free fatty acids, monoglycerides and phospholipids) in the intestinal lumen. Once packed into mixed micelles, carotenoids can be absorbed by the small intestinal epithelium (enterocytes) via

simple diffusion and receptor-mediated transport (Yonekura & Nagao 2007, Parker 1996), where they are packed into triglyceride-rich chylomicrons and transported into blood circulation via the lymphatic system. Carotenoids achieve maximum levels in the plasma within a few hours (e.g., ~5 h for β-carotene) (Parker et al. 1999). Elimination half-life (t(1/2) takes several days. For instance, 5-7 days for β-carotene and 2-3 days for lycopene, respectively (Schwedhelm et al. 2003).

Provitamin A carotenoids (β-carotene, α-carotene and β-cryptoxanthin) are partly converted to vitamin A, primarily retinyl esters, in the intestinal mucosa. Carotenoids can be enzymatically cleaved into vitamin A, if the carotenoid contains an unsubstituted β-ionone ring with a polyene side-chain of at least 11 carbon atoms. Cleavage is catalyzed by an O_2-dependent dioxygenase. Essentially, two retinal molecules produced from carotenoid cleavage are reduced to retinol (Tapiero et al. 2004) (Figure 3). However, in reality, conversion of β-carotene and other provitamin A carotenoids into vitamin A is ineffective (Shils et al. 2006). Vitamin A is an essential micronutrient for cell growth, embryonic development, vision, and immune system function (Jackson et al. 2008).

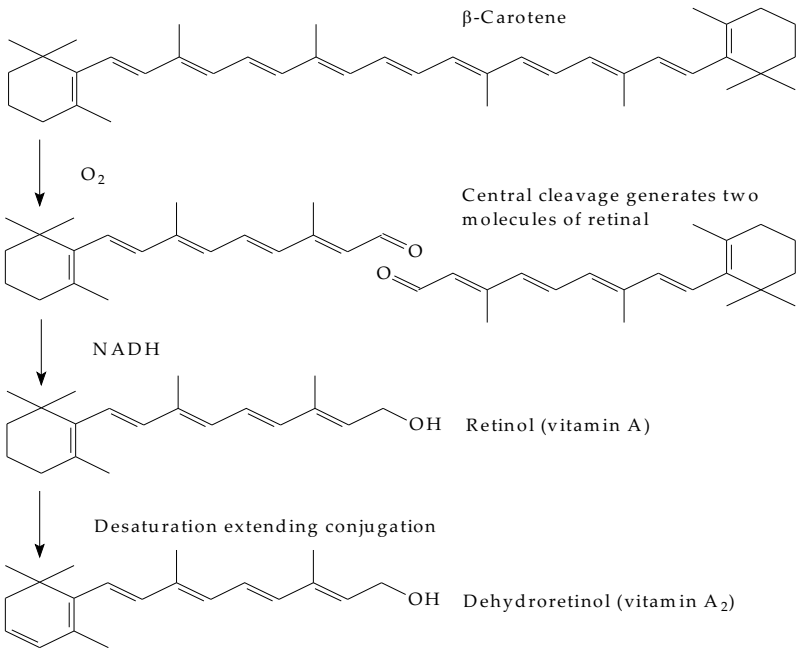

Figure 3. Dietary β-carotene can serve as a precursor for vitamin A (retinol) in humans/mammals. Cleavage is catalyzed by an O_2-dependent dioxygenase, probably via intermediate peroxide. Vitamin A_2 (dehydroretinol) is an analog of retinol containing a cyclohexadiene ring system. Retinol and its derivatives are found only in animal products. NADH = Nicotinamide adenine dehydrogenase.

The chylomicrons are rapidly degraded by lipoprotein lipase in the blood stream. Chylomicron remnants containing carotenoids are rapidly cleared from the plasma

by the liver (Parker 1996, Yeum & Russell 2002). Carotenoids excrete from the liver by binding to very low density lipoprotein (VLDL) (Parker 1996).

Up to 75% of the hydrocarbon carotenoids (α-carotene, β-carotene and lycopene) are bound to LDL, while (53%) the polar dihydroxy carotenoids (e.g., lutein and zeaxanthin) are found predominantly in high density lipoprotein (HDL) and lower proportions in LDL and VLDL (Yeum & Russell 2002; Erdman et al. 1993a). Lipophilic carotenoids are mainly located in the core of the lipoprotein, which may not allow their transfer between lipoproteins at an appreciable rate, whereas the more polar carotenoids, which are mainly present on the surface of lipoproteins, are likely to undergo rapid surface transfer, resulting in a more equal equilibration between LDL and HDL (Parker 1996) (Figure 4).

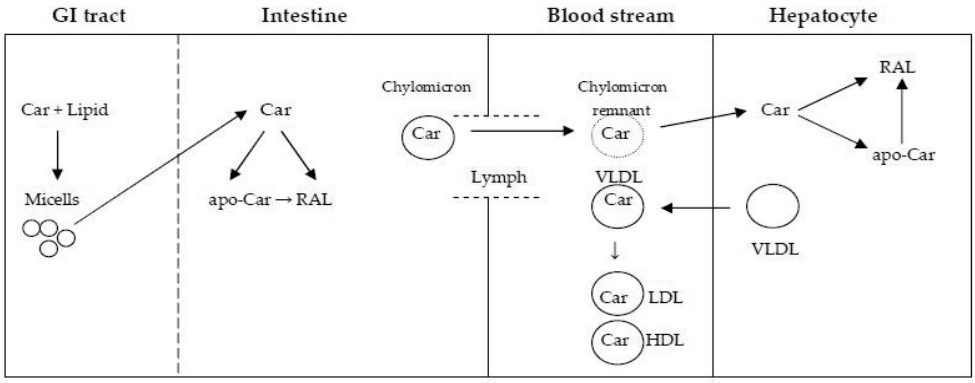

Figure 4. Absorption, metabolism and transport of carotenoids. Abbreviations: CAR, carotenoids; apo-CAR, apo-carotenoids; RAL, retinal; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein}.

Carotenoids are distributed in various tissues, of which adipose tissue is the most important. Lutein and zeaxanthin are the only carotenoids found in human blood that are also found in the macula of the eye (Handelman et al. 1988). The testes, adrenal glands, prostate, breast and liver contain the highest amounts of lycopene (Rao et al. 2006). β-Cryptoxanthin occurs mainly in liver (Kohlmeier & Hastings 1995). β-carotene and α-carotene have been found in the thyroid, kidney, spleen, liver, heart, pancreas, adipose tissue, ovary, adrenal gland and mucosal cells (Stahl et al. 1992). In tissues, carotenoids are thought to be metabolized into small molecules by enzymatic cleavage and/or chemical oxidation with active oxygen species at conjugated double bonds. The hydroxyl group of xanthophylls can be oxidatively metabolized into a carbonyl group (Nagao 2009). For example, the second pathway of β-carotene metabolism is the eccentric cleavage, which occurs at double bonds other than the central 15,15′-double bond of the polyene chain of β-carotene to produce β-apo-carotenals with different chain lengths. The two major sites of β-carotene conversion in humans are the intestine and liver.

2.1.3 Dietary sources and intake

In developed countries, 70–90% of dietary carotenoids come from the intake of fruits and vegetables (Granado et al. 2007). Estimated intakes of carotenoids vary widely between individuals, regions and nations. Studies also report variations between seasons (O'Neill et al. 2001; Elia & Stratton 2005). The majority of carotenoids are derived from a few fruits and vegetables (Granado et al. 1996). Lycopene is found mainly in tomatoes and tomato products, while the principal sources of α-carotene and β-carotene are carrots. Lutein and zeaxanthin exist for example in kale, spinach and maize. The main sources of β-cryptoxanthin are citrus fruits (e.g. oranges) (Osganian et al. 2003). The sources and contents of major carotenoids in selected foods are presented in Table 1.

Table 1. Sources of major carotenoids in selected foods.

Carotenoid	Source	(Content µg/100 g wet wt)
Lycopene	Tomato and tomato products	800-94000
	Red watermelon	3500-13500
	Pink grapefruit	750-3400
	Papaya	7600
	Guava	770-1800
	Rose hip, canned	780
β-Carotene	Carrots	4400-9800
	Apricots	590-3000
	Mangoes	110-1200
	Red pepper	1400-2400
	Kale	1000-7400
	Spinach	3100-4800
	Broccoli	290-1800
α-Carotene	Carrots	2100-5000
	Banana	60-160
	Pumpkin	1900
	Peppers	10-300
	Avocado	17-30
	Apricots	3-40
Lutein and zeaxanthin	Kale	4800-11500
	Spinach	5900-7900
	Broccoli	710-3300
	Peas	1900
	Cress	5600-7500
	Parsley	6400-10700
	Lettuce	1000-4800
	Maize	50-800
	Egg yolk	400-1600
β-Cryptoxanthin	Yellow watermelon	59-100
	Oranges	16-1300
	Papaya	1000
	Mango	20-320
	Red pepper	280-450
	Pineapple	70-120
	Pumpkin	60

Data was taken from O'Neill et al. 2001; Osganian et al. 2003; Maiani et al. 2009 and Granado-Lorencio et al. 2007

There are no recommendations for intake of carotenoids, since carotenoids are not indicated to be essential nutrients for human, unlike vitamin A. Recommended dietary allowances (RDA) exist for vitamin A (Tabacchi et al. 2009). Vitamin A deficiency is known to cause acne, dry hair, fatigue, growth impairment, insomnia, hyperkeratosis (thickening and roughness of skin), immune impairment, night blindness, and weight loss (Underwood 2004). The amount of carotenoids in the diet is difficult to estimate, partly because the methods used for establishing food composition tables are not specific or sensitive enough (Rissanen 2003).

There are major differences in the daily intake of carotenoids between populations. The daily intake of lycopene from tomatoes and other sources has been reported to be 0.8 mg for men in Finland (Ylönen et al. 2003), whereas the intake of lycopene has been found to be 2.1 mg in Spain (Garcia-Closas et al. 2004), 1.2 mg in Netherlands (Männistö et al. 2007), 6.6 mg in the USA (Slattery et al. 2000) and as high as 7.4 mg in Italy (Lucarini et al. 2006). The daily intake of α-carotene and β-carotene has been measured to be 0.15 and 2.6 mg in Italy (Lucarini et al. 2006), 0.3 and 1.1 mg in Spain (Garcia-Closas et al. 2004), 0.7 and 3.0 mg in Netherlands (Männistö et al. 2007) and 1.2 mg and 6.4 mg in the USA (Bandera et al. 1997), respectively. Intakes of β-cryptoxanthin and lutein + zeaxanthin have been identified to be 0.2 and 4.0 mg in Italy (Lucarini et al. 2006), 0.3 and 0.5 mg in Spain (Garcia-Closas et al. 2004), 0.2 and 3.0 mg in the Netherlands (Männistö et al. 2007), respectively. In Finland, the dietary intakes of α-carotene, β-carotene, β-cryptoxanthin and lutein + zeaxanthin were reported to be 0.08–0.5, 1.6–3.5, 0.003–0.025 and 1.0–1.14 mg/d, respectively (Männistö et al. 2007; Ylönen et al. 2003; Montonen et al. 2004; Kleemola et al. 2002).

2.1.4 Carotenoid concentrations in blood circulation

Blood concentrations of carotenoids have shown great variability among different populations (Table 2). In a study covering nine European countries (Al-Delaimy et al. 2004), the plasma concentrations of lutein and zeaxanthin were the highest in Italy and Greece; β-cryptoxanthin was highest in Spanish regions; lycopene tended to be highest in Italy, Spain and Greece and lowest in Sweden. Concentrations of α-carotene or β-carotene did not differ between North and South (Al-Delaimy et al. 2004). Women had generally higher individual carotenoid concentrations in all regions than men. Variation of carotenoid concentrations between regions may be a consequence of different dietary intake of fruits and vegetables and influence of season. It is likely that seasonal fruit and vegetables that are main source of certain carotenoids (tomatoes for lycopene and citrus fruits for β-cryptoxanthin) will have a significant effect on blood levels (lower in the Northern Europe), although influence of season has decreased in industrialised countries (Al-Delaimy et al. 2004). Serum carotenoids have also been assessed in five European countries by Olmedilla et al. (Olmedilla et al. 2001), who similarly have reported wide variability between Northern and Southern Europe. Spain had the highest β-cryptoxanthin concentrations, while lutein and zeaxanthin were higher in Southern

Table 2. Examples of mean concentrations of serum or plasma carotenoids (µmol/l) in the European countries and the USA.

	Lutein		Zeaxanthin		β-cryptoxanthin		Lycopene		α-Carotene		β-Carotene	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Sweden	0.28	0.28	0.06	0.06	0.13	0.20	0.46	0.52	0.11	0.20	0.30	0.54
Finland	-	-	-	-	-	-	0.31	0.30	0.12	0.20	0.51	0.73
Germany	0.36	0.29	0.08	0.08	0.17	0.27	0.69	0.62	0.11	0.23	0.37	0.64
The Netherlands	0.28	0.32	0.07	0.08	0.17	0.27	0.54	0.47	0.08	0.12	0.29	0.37
Denmark	0.28	0.34	0.05	0.07	0.11	0.23	0.58	0.53	0.15	0.22	0.31	0.47
UK	0.26	0.30	0.06	0.07	0.14	0.21	0.72	0.77	0.16	0.24	0.41	0.53
Spain	0.27	0.28	0.11	0.07	0.40	0.42	0.53	0.51	0.07	0.07	0.31	0.34
Greece	0.51	0.52	0.11	0.10	0.33	0.44	0.90	0.87	0.08	0.13	0.40	0.53
Italy	0.61	0.70	0.11	0.11	0.31	0.53	1.29	1.32	0.08	0.19	0.39	0.67
USA	0.27	0.28	0.06	0.06	0.09	0.09	0.76	0.76	0.16	0.22	0.64	0.86

Data was taken from Al-Delaimy et al. 2004; Dwyer et al. 2004; Olmedilla et al. 2001

Europe (France and Spain) than in the North (Northern Ireland and the Republic of Ireland). No clear north–south trend was found for α-carotene or β-carotene (Olmedilla et al. 2001).

Reference ranges for serum/plasma carotenoids have been determined only for lycopene and β-carotene in a few Finnish laboratories. Serum concentrations of carotenoids from a study of five European countries (Spain, France, the Netherlands, Northern Ireland and the Republic of Ireland) may be considered as 'reference values' in the serum of healthy, non-smoking middle-aged subjects (Olmedilla et al. 2001). The reference values determined in various populations are described in Table 3.

2.1.5 Bioactivity

2.1.5.1 Antioxidant activity

Carotenoids have antioxidant activity, which may protect against chronic diseases by decreasing the oxidative damage of cell lipids, lipoproteins, proteins and DNA (Poulsen et al. 2000; Stanner et al. 2004). Astaxanthin has been reported to be a 10-fold stronger antioxidant than β-carotene and 100-fold stronger than α-tocopherol, respectively (Naguib 2000). Oxidative stress has been known to be involved in the initiation and progression of several chronic diseases. Carotenoids principally scavenge two types of ROS: singlet molecular oxygen ($^1O_2$) and peroxyl radicals. They deactivate effectively the electronically excited sensitizer molecules, which are involved in the generation of radicals and singlet oxygen (Young & Lowe 2001). Dietary carotenoids protect human lymphocytes from damage by singlet oxygen $^1O_2$, and may lower the risk for several degenerative diseases, including cancers, cardiovascular or ophtalmological diseases (Zhao et al. 2006; Lornejad-Schafer et al. 2007). The efficacy of carotenoids for physical quenching depends on a number of conjugated double bonds present in the molecule. β-Carotene, zeaxanthin, β-cryptoxanthin, and α-carotene belong to the group of highly active quenchers of $^1O_2$ (Cantrell et al. 2003). Lycopene is a potent antioxidant and the most efficient quencher of $^1O_2$ (Di Mascio et al. 1989). Scavenging of peroxyl radicals generated in the process of lipid peroxidation interrupts the reaction sequence, finally leading to damage in lipophilic compartments. Lycopene was reported to be more effective than β-carotene in cell protection against hydrogen peroxide (H_2O_2) and nitrogen dioxide radicals ($NO^{\bullet}_2$) (Bohm et al. 2001). Due to the unique structure of the terminal ring moiety, the terminal ring of astaxanthin is able to scavenge radicals both at the surface and in the interior of the phospholipid membrane. The unsaturated polyene chain traps radicals in the membrane (Goto et al.2001).

Table 3. Reference values (µmol/l) for main carotenoids in serum of healthy subjects.

	β-Carotene	α-Carotene	Lutein	Zeaxanthin	β-cryptoxanthin	Lycopene	Reference
Finland							
Yhtyneet Medix							(Laboratoriokäsikirja 2009-2010)
laboratoriot	0.28-2.33						
MILA	0.20-2.40					≤ 0.90	(Mineraalilaboratorio MILA)
Spain[a]							
Men	0.067-0.553	0.016-0.146	0.078-0.438	0.020-0.132	0.067-1.005	0.112-0.877	(Olmedilla et al. 1997)
Women	0.087-0.818	0.018-0.225	0.094-0.442	0.010-0.146	0.096-1.443	0.107-0.922	
Whitehall II Study[b]	0.050-2.14						(Armstrong et al. 1997)
France[c]							
Men	0.08-1.53	0.02-0.54	0.11-0.93	0.03-0.51	0.06-0.82	0.09-0.63	(Olmedilla et al. 2001)
Women	0.23-2.05	0.04-0.96	0.19-1.00	0.04-0.34	0.08-0.91	0.13-1.13	
Northern Ireland							
Men	0.08-1.59	0.0-0.18	0.07-0.37	0.01-0.18	0.01-1.24	0.09-0.66	
Women	0.13-1.12	0.03-0.28	0.08-0.37	0.02-0.18	0.05-0.90	0.11-0.71	
Republic of Ireland							
Men	0.07-1.11	0.01-0.29	0.07-.036	0.01-0.18	0.0-0.48	0.05-1.30	
Women	0.17-1.13	0.02-.028	0.09-0.44	0.01-0.12	0.03-0.31	0.07-0.91	
The Netherlands							
Men	0.11-0.92	0.01-0.26	0.07-0.42	0.01-0.15	0.02-1.21	0.06-0.95	
Women	0.12-1.03	0.03-0.34	0.08-0.51	0.01-0.20	0.10-1.31	0.02-1.16	
Spain							
Men	0.04-.096	0.02-0.24	0.14-0.67	0.03-0.21	0.16-1.41	0.08-0.52	
Women	0.07-0.94	0.02-0.24	0.12-0.82	0.04-0.16	0.11-1.12	0.09-0.91	

[a]Values between 5 and 95 percentiles, [b]The non-parametric 95% reference interval, [c]Range

2.1.5.2 Carotenoids as prooxidants

Burton and Ingold (1984) demonstrated first that at high non-physiological oxygen pressure (pO_2 760 mmHg) at a concentration of 500 mM β-carotene has prooxidant behaviour (Burton & Ingold 1984). The same behaviour was confirmed for β-carotene by Palozza et al. (1997).

Interacting with ROS or reactive nitrogen species (RNS), the carotenoid molecule is oxidized and/or cleaved to generate products that themselves possess different, possibly deleterious, activity in biological systems. The presence of high cellular concentrations of carotenoid alters the properties of a biological membrane and may increase its permeability to toxins or free radicals. In this particular case, different carotenoids would be expected to behave quite differently, as they are incorporated into membranes differently. This may also alter their ability to interact with ROS or other antioxidants. Interaction with ROS results in the formation of a carotenoid peroxyl radical, which itself initiates further lipoperoxidation. The formation of this potentially highly reactive species may be the consequence of a high carotenoid concentration and/or increased oxygen tensions (Lowe et al. 2003).

2.1.5.3 Regeneration of carotenoids

It is known that carotenoids can be regenerated from their radical cations formed during oxidative stress by reacting with tocopherols and tocotrienols (Mortensen & Skibsted 1997) as described below:

$$Car^{\bullet+} + TOH \rightarrow Car + TO^{\bullet} + H^{+}$$

It is possible that tocopherols may react with the carotenoid radical cations through other means, which may explain why only partial recovery of carotenoids is observed (Mortensen & Skibsted 1997).

$$Car^{\bullet+} + TOH \rightarrow [Car{-}TO]^{\bullet} + H^{+}$$

Recently, isoflavonoid dianions have shown to regenerate carotenoids from their radical cationic form. Electron transfer to radical cations of β-carotene, zeaxanthin, canthaxanthin, and astaxanthin was found to depend on carotenoid structures and more significantly on the deprotonation degree of the isoflavonoids. Electron transfer from isoflavonoids to the carotenoid radical cation, as formed during oxidative stress, is faster for the astaxanthin radical than for the other carotenoids (Han et al. 2010). Anionic forms of the conjugated bases of baicalin have also been found to regenerate the radical cation of β-carotene (Liang et al. 2009). Carotenoids can also regenerate each other. It has been shown that lutein and zeaxanthin are recycled by lycopene. Recycling is more efficient for lycopene than for β-carotene because lycopene is higher in the antioxidant hierarchy (Mortensen & Skibsted 1997).

2.1.5.4 Free radicals

Free radicals are atomic or molecular species that have one or more unpaired electrons in the outer orbital, making them highly reactive. Free radicals can oxidatively damage nucleic acids, lipids, proteins and carbohydrates (McCall & Frei 1999), thereby contributing to a number of human degenerative chronic diseases, including atherosclerosis, cancers and cataracts. However, many chemical reactions of free radicals form part of the basic chemical processes of normal human metabolism, such as the regulation of vascular tone, antimicrobial killing, and regulation of cellular proliferation and growth (Mugge 1998). The human body possesses specific defense mechanisms to protect against excess formation of free radicals and from tissue injury. The enzymes, superoxide dismutase (SOD), catalase, glutathione peroxidase (de Groot 1994), paranoxonase (Aviram et al. 1998), and antioxidants, such as α-tocopherol, carotenoids, vitamin C, urate and thiols, scavenge effectively free radicals, thus protecting the body from oxidative damage (de Groot 1994).

Most of the free radicals are derived from molecular oxygen. The term often used is ROS. This term includes radicals as well as chemicals that can take part in radical type reactions (i.e., gain or loose electrons) but are not true radicals in that they do not have unpaired electrons but are often involved in the generation of free radicals. Examples of non-radical ROS include H_2O_2, hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1O_2$) (Pryor & Squadrito 1995; Beckman & Koppenol 1996). In addition to oxygen-based radicals, there are also reactive nitrogen species (RNS), such as nitric oxide (NO) and nitrogen dioxide (NO_2) (Darley-Usmar & Halliwell 1996).

2.1.5.5 Carotenoid reactions with free radicals

Carotenoids are known to lose their color when they react with free radical species. This phenomenon is explained by degradation of the polyene chain or the addition of double bonds. There are at least three possible mechanisms for the reaction of carotenoids with radicals in (Krinsky & Yeum 2003): (1) radical addition, (2) electron transfer to the radical or (3) allylic hydrogen abstraction.

1) Radical addition: Burton and Ingold (Burton & Ingold 1984) first proposed the addition reaction. They suggested that a lipid peroxyl radical ($ROO^{\bullet}$) might add at any place across the carotenoid (CAR) polyene chain, resulting in the formation of a carbon-centered radical ($ROO–CAR^{\bullet}$). Since this radical would be resonance-stabilized, it would interfere with the propagating step in lipid peroxidation and would explain the many examples of the antioxidant effect of carotenoids in solution (Palozza & Krinsky 1992). The proposed reaction is described in reaction (1).

$$CAR + R^{\bullet} \rightarrow R–CAR^{\bullet} \qquad \text{(Reaction 1)}$$

2) Electron transfer: reactions of this type have been reported, resulting either in the formation of a carotenoid radical such as the cation radical $CAR^{+\bullet}$ the anion radical, $CAR^{-\bullet}$, or in the formation of an alkylradical, $CAR^{\bullet}$. For example, when lycopene reacts with the superoxide radical ($O_2^{-\bullet}$), electron transfer occurs with the formation of the anion radical, $CAR^{-\bullet}$ (Conn et al. 1992).

$$CAR + R^{\bullet} \rightarrow CAR^{\bullet +} + R^{-} \qquad \text{(Reaction 2)}$$

3) Hydrogen abstraction: this process has been suggested by Woodall et al. (Woodall et al. 1997).

$$CAR + R^{\bullet} \rightarrow CAR^{\bullet} + RH \qquad \text{(Reaction 3)}$$

ROS are generated endogenously through normal metabolic process, life style activities, and diet. They react with critical cellular biomolecules such as lipids, proteins and DNA and initiate events that lead to increased risk of chronic disease such as cancer, cardiovascular disease, and osteoporosis (Buico et al. 2009; Milde et al. 2004).

2.2 CAROTENOIDS AND LIPIDS

2.2.1 The role of oxidized LDL in atherogenesis

LDL is comprised 78% of lipids and 22% of proteins. The main protein molecule in LDL is apoliporotein B (apoB). Phospholipids and free cholesterol align on the surface of a particle, while triglycerides and cholesteryl ester are packed in the core of the particle (Itabe 2009; Hevonoja et al. 2000). Under oxidative stress, lipid molecules containing polyunsaturated fatty acids (PUFA) in LDL are oxidized. During the oxidation process, a variety of lipid oxidation products are formed and the apoB protein is oxidatively modified, as well. Radical chain reactions on PUFA lead to the formation of conjugated dienes and lipid hydroperoxides, and secondary cleavage reactions produce a variety of aldehyde compounds, such as malondialdehyde (MDA) (Steinberg 2009; Itabe 1998).

Oxidative modification of LDL is involved in the early development of atherosclerotic lesions through the formation of macrophage-derived foam cells. It has been proposed that foam cells could be induced from macrophages after taking up oxidatively modified LDL (ox-LDL). Macrophages contain receptors for ox-LDL which can bind and take up ox-LDL. There are more than ten receptors for ox-LDL in macrophages known as scavenger receptors (Itabe 2003).

It has been shown that the plasma levels of oxidized LDL are higher in patients with cardiovascular diseases, such as acute myocardial infarction (AMI). In addition, increased levels of oxidized LDL were found in patients with carotid atherosclerosis (Ehara et al. 2001). A recent in vivo study has provided evidence

that ox-LDL can be used to predict future atherosclerotic events (Naruko et al. 2006). It has been suggested that high ox-LDL levels in the acute phase of AMI could be caused by a massive release of ox-LDL from the ruptured plaques, whereas high levels during the stable phase may indicate that the patients have strong sources of ox-LDL production. Such sources may include exposure to strong oxidative stress or the presence of unstable plaques somewhere in blood vessels that release ox-LDL from the lesions (Itabe 2009).

2.2.2 Effect of carotenoids on oxidative modification of lipids

The effect of carotenoids on lipid peroxidation has been studied in cell cultures (Table 4). These studies used much higher carotenoid concentrations than the normal physiological concentrations in human blood circulation. Lipid oxidation was followed by e.g., copper or 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) induced oxidation (susceptibility to oxidation expressed as lag time) or formation of thiobarbituric acid reactive substances (TBARS). During lag time LDL is depleted of its antioxidants and after that the rate of lipid peroxidation is rapidly accelerated (Schreier et al. 1997). These studies are inconsistent, possibly because of various concentrations of carotenoids and use of various oxidation inducers. In addition, copper ion concentration in the reaction mixture varied by study. There are no generally recommended reference methods for lipid oxidation measurement. Thus, each laboratory can modify the measurements which may cause variation in the results.

There are numerous intervention studies concerning supplementation of healthy subjects with carotenoids (capsules) or food stuffs (e.g., tomato products), in which effects of carotenoids on lipid oxidation are evaluated (Table 5 and Table 6). Oxidative modification of lipids was followed e.g., by copper/AAPH induced oxidation (susceptibility to oxidation expressed as lag time) or measuring TBARS, F_2 free isoprostanes or MDA. Copper or AAPH -induced methods are in vitro lipid oxidation measurements, whereas in vivo formed F2 isoprostanes or MDA reflect better the lipid oxidation in the body. The traditionell, but quite unspecific, TBARS measurement, in principle, is an in vivo measurement, but the high temperature needed for the reaction may cause in vitro formation of TBARS in the sample. Of the four latest placebo controlled studies, two showed decreasing lipid oxidation after astaxanthin or combined β-carotene, lycopene and lutein supplementation, but in two controlled studies and in uncontrolled studies there were no effect (Table 5). Neither effect has been found in the latest controlled dietary supplementation studies (Table 6). However, most of the dietary supplementation studies have been uncontrolled leading to quite uncertain results. It seems that most of intervention studies studies do not support the protective role of carotenoid supplementation against lipid oxidation.

Table 4. The effect of carotenoids on oxidation of LDL in cell culture studies.

Model	Carotenoid	Concentration	Measurement	Effect on LDL oxidation	Reference
LDL in cell-free system and in human monocyte macrophages (Ham's F-10 medium)	β-carotene	2 µmol/l	TBARS[a] and Lag time[b] (2.5 µM Cu^{2+})	↓	(Jialal et al. 1991)
Isolated human LDL	β-carotene	2.79 nmol/mg	Lag time (1.25 µM $Cu^{2+}$and 400 mM AAPH[c])	No effect	(Gaziano et al. 1995)
LDL in human monocyte macrophages (Ham's F-10 medium)	β-carotene	4.7 µmol/l	TBARS	↓	(Carpenter et al. 1997)
	Canthaxanthin	22.1 µmol/l		↓	
	Zeaxanthin	44 µmol/l		↓	
LDL in human endothelial cells (Ham's F-10 medium)	β-carotene	160 µmol/l	TBARS and FOX[d]	↓	(Dugas et al. 1998)
	Lutein	40 µmol/l		↑	
	Lycopene	60 µmol/l		↑	
Isolated human LDL	β-carotene	Study 1: 0.25-0.97 µg/mg Study 2: 0.17-10 µg/mg Study 3: 0.14-8 µg/mg	TBARS and Lag time (40 µM Cu^{2+})	↑	(Bowen & Omaye 1998)
Isolated human LDL	Astaxanthin	21-83.8 µmol/l	Lag time (400 µM MeO-AMVN)[e]	↓	(Iwamoto et al. 2000)

Resistance to isolated LDL to copper/AAPH induced oxidation measured by spectrophotometer. [a] Thiobarbituric acid reactive substances, [b]Time to maximal oxidation rate., 2,2-azobis(2-amidinopropane) dihydrochloride, [d] Ferrous oxidation/xylenol orange, [e] 4-methoxy-2,4-dimethylvaleronitrile, ↓ Decrease oxidative modification of LDL,↑ Increase oxidative modification of LDL

Table 5. Effect of carotenoid supplementation on LDL oxidation in healthy humans. Latest most important studies.

Carotenoid supplementation	Dose (mg/d) and duration	N	Measurement	Effect on LDL oxidation	Reference
β-Carotene (uncontrolled)	50-100 mg for 3 weeks	16	Lag time[a] (1.25 μM $Cu^{2+}$and 4 μM AAPH[b])	No effect	(Gaziano et al. 1995)
Lycopene β-Carotene α-Carotene (Placebo controlled)	13.3 mg 8.2 mg 3.7 mg for 12 weeks	51	Lag time (15 μM Cu^{2+})	No effect	(Carroll et al. 2000)
Astaxanthin (Placebo controlled)	1.8-21.6 mg for 14 days	24	Lag time (400 μM MeO-AMVN)[c]	↓	(Iwamoto et al. 2000)
β-Carotene Lycopene Lutein (Placebo controlled)	15 mg 15 mg 15 mg for 3 months	175	Lag time (2.5 μM Cu^{2+})	No effect	(Hininger et al. 2001)
β-Carotene α-Carotene Lycopene Lutein (Placebo controlled)	6 mg 1.4 mg 4.5 mg 4.4 mg for 3 weeks	32	Lag time (5 μM Cu^{2+})	↓	(Kioskias & Gordon 2003)
β-Cryptoxanthin (uncontrolled)	1.3 mg for 24 h	12	Lag time (20 μM Cu^{2+})	No effect	(Wolters & Hahn 2004)

Resistance to isolated LDL to copper/AAPH induced oxidation measured by spectrophotometer. [a]Time to maximal oxidation rate, [b] 2,2-azobis(2-amidinopropane) dihydrochloride, [c] 4-methoxy-2,4-dimethylvaleronitrile, ↓ Decrease oxidative modification of LDL, N = number of subjects

Table 6. Summary of latest dietary intervention studies in healthy humans.

Dietary Supplementation	Dose and duration	N	Measurement	Effect on lipid oxidation	Reference
Tomato juice Tomato sauce Tomato oleoresin (Cross-over)	One week of each	19	Lag time[a] (Cu^{2+}) LDL-TBARS[b]	↓	(Agarwal & Rao 1998)
Lycopene, β-carotene and lutein rich foods (Cross-over)	~300-400 g 4 weeks	34	Lag time (11.7 μM Cu^{2+})	↓	(Chopra et al. 2000)
Vegetables, berries and apples (Control group)	170-815 g (lutein, cryptoxanthin, lycopene, α-carotene and β-carotene) for 6 weeks	77	Plasma and LDL-TBARS Lag time (5 μM Cu^{2+}) F_2 isopostanes[c]	No effect	(Freese et al. 2002)
Tomato products and guava (Cross-over)	~585 g for 14-16 days	31	Lag time (1 mM Cu^{2+})	↓	(Ahuja et al. 2003)
Tomato products with olive oil (Cross-over)	Fresh tomato 100 g Sauce 60 g Paste 15 g each for 3 weeks	12	Lag time (5 μM Cu^{2+}) F_2 isoprostanes[c]	↓	(Visioli et al. 2003)
Tomato or carrot juice (Cross-over)	330 ml for 2 weeks	22	Lag time (20 μM Cu^{2+}) MDA[d]	No effect	(Briviba et al. 2004)
Soups and beverages of fruits and vegetables (Control group)	400 g for 4 weeks	36	Lag time (1.66 μM Cu^{2+})	No effect	(Paterson et al. 2006)
Lutein + green tea extract (Cross-over)	12 mg 200 mg for 4 weeks	40	MDA	No effect	(Li et al. 2010)

Resistance to isolated LDL to copper induced oxidation measured by spectrophotometer. [a] Time to maximal oxidation rate, [e] Thiobarbituric acid reactive substances, [c] Lipid peroxidation products of arachidonic acid, [d] Malondialdehyde, ↓ Decrease oxidative modification of lipids, N = number of subjects

2.2.3 Epidemiological evidence on cardiovascular diseases

Over the last 20 years, many interesting studies have been published concerning an association between the intake of carotenoids and risk of cardiovascular diseases (CVD). The largest studies include the Health Professionals Follow-Up Study

(HPFS) (Rimm et al. 1993), a study of approximately 40000 male health professionals, and the Nurse's Healthy Study (NHS) (Osganian et al. 2003), a study of approximately 80000 female nurses. The HPFS study found an inverse association with CVD risk among current smokers (RR=0.30; 95% CI: 0.11–0.82) by comparing the highest quintile of dietary carotene intake to the lowest. For former smokers, RR was 0.6, and the risk was nonsignificant for nonsmokers. In the NHS, the relationship between dietary intake of carotenoids and the risk of coronary artery disease (CAD) was studied in 73286 female nurses using a semi-quantitative food-frequency questionnaire. During the 12-year follow-up, they identified 998 incident cases of CAD and modest, significant, inverse associations between the highest quitiles of β-carotene (7.6 mg) and α-carotene (1.5 mg) intakes and the risk of CAD was observed, but not with lutein plus zeaxanthin, lycopene, or β-cryptoxanthin intakes.

The large Physicians' Health Study identified 499 cases of CVD during a mean follow-up of 2.1 years. Plasma lycopene was not associated with a risk of CVD in middle-aged and older men (RR=1.03; 95% CI: 0.65–1.64) (Sesso et al. 2005), whereas plasma lycopene was inversely related to a risk of CVD in women (Sesso et al. 2004). In a case-control study in Milan, Italy, examining 760 patients with nonfatal acute myocardial infarction (AMI) and 682 controls, the risk of AMI decreased with increasing daily intake of α-carotene (684 μg), β-carotene (4186 μg) and β-cryptoxanthin (340 μg). No associations, however, were observed for total carotenoids, lycopene, lutein plus zeaxanthin and retinol (Tavani et al. 2006).

Fifteen cohort studies were included in a meta-analysis consisting of 7415 incident coronary heart disease (CHD) cases and 374,488 participants with a median follow-up of approximately 10, 8.5, and 15 years for intake from diet or supplements of vitamins C, E, and β-carotene, respectively. The combined RR in the highest tertile of β-carotene intake as compared to the lowest tertile for CHD was 0.78 (95% CI: 0.53–1.04). Findings of the meta-analysis suggested that an increase in the dietary intake of β-carotene has encouraging prospects for possible CHD prevention (Ye & Song 2008). In another pooled cohort analysis, during a 10-year follow-up, 4647 major incident CHD events occurred in 293172 subjects free from CHD at baseline. Of the individual carotenoids, lutein intake was significantly inversely associated with CHD incidence (RR=0.89; 95% CI: 0.75–1.04; *P* for trend=0.03), whereas the strength of association for the other carotenoids was nonsignificant. The results suggested that a higher dietary intake of lutein reduces the risk of CHD (Knekt et al. 2004).

In a recent study, it was determined whether low plasma carotenoids are associated with increased all-cause mortality among older adults. In a multivariate Cox proportional hazards model, adults in the highest tertile of plasma carotenoids at enrollment had lower mortality compared to those in the lowest tertile (Hazards Ratio 0.81, 95%; CI: 0.65–0.99; *P* for trend = 0.046). The results suggested that low plasma carotenoids are an independent risk factor for mortality among older adults (Lauretani et al. 2008). In the Zutphen Elderly Study in the Netherlands,

dietary intakes of α-carotene and β-carotene were inversely associated with CVD mortality in elderly men after a 15-year follow-up. Intake of α- and β-carotene was associated with ~20% lower risk ratio (RR) of death from CVD. The multivariate-adjusted RR was 0.72 (95% CI: 0.57–0.90) for α-carotene and 0.74 (0.60–0.91) for β-carotene. The RR for the consumption of carrots was 0.83; 95% CI: 0.68–1.00 (Buijsse et al. 2008).

2.3 CAROTENOIDS AND CANCER

2.3.1 The role of carotenoids in carcinogenesis

Carcinogenesis is the process by which normal cells are transformed into cancer cells. Mutations in DNA leading to cancer disrupt the balance between proliferation and programmed cell death (apoptosis). A series of several mutations in certain classes of genes is usually required before a normal cell transforms into a cancer cell. The uncontrolled and often rapid proliferation of cells can lead to benign tumors, some of which may turn into malignant tumors (Kleinsmith 2006). Although lifestyle and dietary factors are known to be important in preventing cancer, the mechanism still remains unclear (Kushi et al. 2007; Correa Lima & Gomes-da-Silva 2005). ROS produced in cells, largely as a consequence of metabolic processes, constantly threatens the integrity and correct functioning of cellular DNA (Lindahl 1993). Several oxidants have the capacity to produce pro-mutagenic lesions in DNA (Shibutani et al. 1991), which may play a significant role in the development of cancer.

Carotenoids may protect against carcinogenesis by scavenging of DNA-damaging free-radicals, induce enzymes (e.g., cytochrome P450) that are involved in the metabolism of several carcinogens, affect on cell proliferation, cell differentiation, gap-junctional cell communication and enhance the immune system (Astorg 1997, Bhuvaneswari & Nagini 2005). It has been shown that at physiological concentrations, lycopene inhibit the growth of normal human prostate epithelial cells in vitro, which may prevent a benign prostate hyperplasia, a risk factor for prostate cancer (Obermuller-Jevic et al. 2003). In a previous study, lycopene and hexane extract of tomato paste inhibited cell gwroth in a dose-dependent manner (0.1-50 μmol/l of lycopene) (Hwang & Bowen 2005). Interference in androgen metabolism, and inhibition of growth factors and cytokine activity, appear to be the major pathways through which lycopene inhibits prostate and breast cancer growth (Karas et al. 2000).

In addition to lycopene, other carotenoids, such as lutein α-carotene and β-cryptoxanthin have shown a strong anti-carcinogenic activity in animal screening tests (Nishino et al. 2009). α-Carotene has shown higher activity than β-carotene in suppressing tumorigenesis in skin, the lungs, liver and colon. In the skin tumorigenesis experiment, the average number of tumors in per mouse was significantly smaller in the α-carotene group than in the control group. β-Carotene treatment also decreased the development of tumors, though the difference

between the treatment and control groups was not significant (Nishino et al. 2009). The ferret and mouse lung cancer models have been used to investigate the mechanism of interaction of β-carotene with carcinogens in the lung. No effects or even protective effects against smoke or carcinogen exposure were observed when β-carotene was applied at physiological doses or in combination with vitamins C and E (Goralczyk et al. 2009). Lorenzo et al. (Lorenzo et al. 2009) reported a clear, dose-dependent antioxidant-protective effect for β-cryptoxanthin that decreased the damage to DNA induced by H_2O_2.

2.3.2 Carotenoid supplementation and cancer biomarkers

Supplementation of carotenoids may have beneficial effects against cancer development and progression, although the results have been inconsistent. A pilot study investigated the effects of lycopene supplementation in elderly men diagnosed with benign prostate hyperplasia (BPH), who were at increased risk of developing prostate cancer. The 6-month lycopene supplementation (15 mg/d) decreased prostate specific antigen (PSA) levels, whereas no change was identified in the placebo group. The study indicated that lycopene may inhibit disease progression and may ameliorate symptoms in BPH patients (Schwarz et al. 2008). Lycopene intake before prostatectomy has been reported to decrease serum concentrations of PSA and growth of tumors in patients with clinically localized prostate cancer (Kucuk et al. 2001; Chen et al. 2001). A dose of β-carotene (50 mg) on every second day had neither significant effect on the molecular markers of lung carcinogenesis (cytochrome p450 enzyme 1A1 (CYP1A1)) nor on the risk of developing lung cancer (Liu et al. 2009).

2.3.3 Epidemiological evidence on cancer

Of all the cancers, the effect of lycopene on the reduction of prostate cancer has received the most attention (Rao & Rao 2007) (Table 7). An inverse relationship between tomato intake and the risk of prostate cancer was first reported by Giovannucci et al. (Giovannucci et al. 1995). In two previous studies (in Table 7), an inverse association between blood lycopene concentration and the risk of prostate cancer has been observed (Lu et al. 2001; Zhang et al. 2007). Results from a meta-analysis of 11 case-control studies and 10 cohort studies reported a 10% to 20% reduction in prostate cancer risk, though this effect was restricted especially to above 200 g/d of tomato intake (Etminan et al. 2004). Seren et al. 2008 have proposed in their review that the association between the reduced risk of cancer and intake of tomatoes, tomato-based products and blood levels of lycopene is strongest for prostate cancer. The U.S. Food and Drug Administration (FDA) have found only limited credible evidence for tomato intake and a reduced risk of prostate cancer, but not for lycopene intake (Kavanaugh et al. 2007).

Table 7. Summary of epidemiological studies of the relation between circulating levels or intake of lycopene and risk of prostate cancer.

Study population	Type of study	N (Subjects)	Mean/median concentration (µmol/l)[a]	RR/OR (95% CI) for highest catergory vs. lowest, P for trend	Comparison (µmol/l)	Reference
Japanese-Americans in Hawaii	Nested case-control	284	0.25	1.10 (0.50 – 2.20), *P* = 0.86	highest quartile vs. lowest	(Nomura et al. 1997)
Physicians' Health Study	Nested case-control	1872	0.71	0.75 (0.54 – 1.06), P = 0.12	>1.08 vs. <0.49	(Gann et al. 1999)
MSKCC[b], New York	Case-control	197	0.27	0.17 (0.04 – 0.78), P = 0.005	>0.40 vs. <0.18	(Lu et al. 2001)
Atlanta, Detroit, 10 NJ counties	Case-control	437	0.31	0.65 (0.36 – 1.15), P = 0.09	>0.46 vs. <0.19	(Vogt et al. 2002)
Washington county, Maryland	Cohort (1)	546	0.66	0.83 (0.46 – 1.48), P = 0.72	>1.02 vs. <0.40	(Huang et al. 2003)
Washington county, Maryland	Cohort (2)	426	0.76	0.79 (0.41 – 1.54), P = 0.49	>1.17 vs. <0.45	(Huang et al. 2003)
Health Professionals Study	Case-control	900	0.72	0.48 (0.26 – 0.89), P = 0.06	highest quintile vs. lowest	(Wu et al. 2004)
American	Meta-analysis	3968	NR	0.74 (0.59 – 0.92)	highest quintile vs. lowest	(Etminan et al. 2004)
Houston, Texas	Case-control	170	0.49	1.30 (0.63 – 2.71)	>0.49 vs. <0.49	(Chang et al. 2005)
European[c]	Case-control	2060	0.50	0.97 (0.70 – 1.34), P = 0.41	>0.92 vs. <0.28	(Key et al. 2007)
10 centres in USA	Nested case-control	1536	1.18	1.14 (0.82 – 1.58), P = 0.28	>2.02 vs. <0.57	(Peters et al. 2007)
Arkansas	Case-Control	390	0.55	0.45 (0.24 – 0.85), P = 0.042	>0.96 vs. <0.26	(Zhang et al. 2007)
			Mean/median intake (µg/d)		**Comparison (µg/d) or servings/d**	
American	Cohort	773	-	0.65 (0.44 – 0.95), P = 0.01	>10 vs. <1.5/wk tomato-based products	(Giovannucci et al. 1995)
	Cohort	773	-	0.66 (0.49 – 0.90), P = 0.001	2-4 vs 0/wk tomato sauce	(Giovannucci et al. 1995)
Dutch	Cohort	2167	1050	0.98 (0.71 – 1.34), P = 0.58	>2000 vs. <100	(Schuurman et al. 2002)
Italian	Case-control	2745	7487	0.94 (0.72 – 1.23)	highest quintile vs. lowest	(Bosetti et al. 2004)
Chinese	Case-control	404	5061	0.18 (0.08 – 0.41), P = 0.009	>4916.7 vs. <1608.6	(Jian et al. 2005)
American	Cohort	29361	11511	0.95 (0.79 – 1.13), P = 0.33	>17593 vs. <5052	(Kirsh et al. 2006)
				0.90 (0.75 – 1.08), P = 0.14[d]	>12647 vs. <3009	(Kirsh et al. 2006)

[a] Includes cases and controls (serum / plasma), [b] Memorial Sloan-Kettering Cancer Centre, [c] Participants were from Denmark, Germany, Greece, Italy, Netherlands, Spain, Sweden and the United Kingdom, [d] Lycopene from processed sources, NR = not reported, N = number of subjects

A recent review suggested a strong protective effect of lycopene intake and plasma levels of lycopene against gastric and colon cancer, and a weaker effect for cancers of the pancreas and oral cavity (Seren et al. 2008). The FDA found no credible evidence to support an association between tomato or lycopene intake and a reduced risk of lung, colorectal, cervical, gastric, breast, ovarian, endometrial, or pancreatic cancer. The FDA found very limited evidence to support an association between tomato consumption and reduced risks of ovarian, gastric, and pancreatic cancers (Kavanaugh et al. 2007). A recent report by the World Cancer Research Fund stated that foods containing lycopene probably protect against prostate cancer (World Cancer Research Fund/American Institute for Cancer Research 2007).

Two of the latest three meta-analyses found an association between decreased risk or renal or lung cancer and dietary intake of carotenoids or carotenoid containing food stuff (Table 8).

In Table 9, we can see that α-carotene, β-carotene and lycopene are the carotenoids, which are most abundant in plasma sample and thus most often used for risk assessment in epidemiological studies. It has been shown that plasma β-carotene was inversely associated with the risk of gastric cancer (Persson et al. 2008) and colorectal cancer (Jiang et al. 2005). In another study, plasma β-cryptoxanthin and zeaxanthin were associated with reduced risk of gastric adenoma (Jenab et al. 2006). In American case-control study, plasma carotenoids except lycopene were inversely associated with the risk of bladder cancer (Hung et al. 2006). However, in a previous study, high plasma lycopene concentrations were associated with decreased mortality of oral, pharynx or larynx cancers (Mayne et al. 2004). Meta-analysis showed that serum carotenoids may not protect from the risk of lung cancer (Gallicchio et al. 2008).

In a carotenoid supplementation studies (Table 10), the risk of total cancer decreased in men by using a combination of vitamin C, vitamin E and β-carotene (Galan et al. 2005). Interestingly, previous studies have observed either increased risk or no effect on various cancers using§ combinations of carotenoids.

2.4 ANALYSIS OF CAROTENOIDS

2.4.1 Sample collection and storage

There are many reports dealing with the stability of carotenoids in biological samples. Su et al. (Su et al. 2002) observed that there were no significant changes in the concentrations of lutein/zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene, except for lycopene, in whole blood stored at +4°C in the dark. At room temperature, during exposure to light, the concentrations of carotenoids still remained stable, though the reduction in lycopene concentration was significant. All carotenoids were unstable at +35°C. The chemical structure of carotenoids makes them potentially susceptible to oxidative damage or isomerization with exposure to oxygen, light, or heat (Clark et al. 2004). It has been reported that

Table 8. Summary of epidemiological studies of the relation between dietary intake of carotenoids and risk of various cancers.

Study population	Type of study	Dietary intake at baseline and duration	N	Effect on cancer RR/OR (95% CI), P-value	Comparison	Reference
American Massachusetts	FFQ follow up	Green and yellow vegetables for 4.75 y	1271	Risk of total cancer mortality decreased. All vegetables: 0.3 (0.1-0.96) Tomatoes: 0.5 (0.3-0.8)	≥ 2.2 vs. < 0.7 servings/d	(Colditz et al. 1985)
Pooled	Meta-analysis (FFQ)	α-carotene β-carotene β-cryptoxanthin Lutein+zeaxanthin Lycopene for 6-20 y	702647	There were no association between intakes of specific carotenoids and colorectal cancer risk.	Highest quintile vs. lowest	(Männisto et al. 2007)
Pooled	Meta-analysis	α-carotene β-carotene Lycopene β-cryptoxanthin Lutein+zeaxanthin for 4-25 y	878-121700	Total carotenoids decreased risk of lung cancer: 0.79 (0.71-0.87), P<0.001. Individual carotenoids had no effect.	Highest vs. lowest	(Gallicchio et al. 2008)
Pooled	Meta-analysis (FFQ or dietary history) for 7-20 y	Fruits and vegetables and carotenoids	774952	Fruits and vegetables decreased risk of renal cell cancer: 0.68 (0.54-0.87), P_{trend}=0.001 β-carotene decreased risk of renal cell cancer: 0.82 (0.69-0.98) P_{trend}=0.01	Highest quintile vs. lowest ≥ 600 vs. < 200 g/d fruits and vegetables	(Lee et al. 2009b)

FFQ = Food frequency questionnaire, N = number of subjects

Table 9. Summary of the latest epidemiological studies of the relation between circulating levels of carotenoids and risk of various cancers.

Study population	Type of study	Mean/median concentration (µmol/l) at baseline and duration	Subjects N	Effect on cancer RR/OR (95% CI), P-value	Comparison (µmol/l)	Reference
American	Cohort	α-carotene: 0.0.62 β-carotene: 0.34 Lycopene: 0.57 Lutein+zeaxanthin: 0.024 for 90 months	259	Low plasma lycopene concentration was associated with increased mortality of oral, pharynx or larynx cancers.	α-carotene: >0.048 vs. <0.048 β-carotene: >0.22 vs. <0.22 Lycopene: >0.52 vs. <0.52 Lut+zea: >0.25 vs. <0.25	(Mayne et al. 2004)
Japanese	Case-control	α-carotene: 0.08 β-carotene: 0.35 Lycopene: 0.19 β-cryptoxanthin: 0.17 Lutein+zeaxanthin: 0.77 for 6 y	319	In men, plasma α-carotene, β-carotene and total carotenoids associated inversely with the risk of colorectal cancer:	α-carotene: >0.13 vs. <0.055 β-carotene: >0.70 vs. <0.24 Lycopene: >0.35 vs. <0.11 β-cryptoxanthin: >0.33 vs. <0.11 Lut+zea: >1.04 vs. <0.58	(Jiang et al. 2005)
American	Case-control	α-carotene: 0.081 β-carotene: 0.33 Lycopene: 0.26 β-cryptoxanthin: 0.096 Lutein: 0.12 Zeaxanthin: 0.06 for 4 y	446	Plasma carotenoids except for lycopene were inversely associated with the risk of bladder cancer.	α-carotene: >0.097 vs. <0.036 β-carotene: >0.37 vs. <0.12 Lycopene: >0.33 vs. <0.25 β-cryptoxanthin: >0.28 vs. <0.042 Lutein: >0.16 vs. <0.062 Zeaxanthin: >0.071 vs. <0.030	(Hung et al. 2006)
10 European Countries EPIC study[e]	Nested case-control	α-carotene: 0.12 β-carotene: 0.35 Lycopene: 0.51 β-cryptoxanthin: 0.20 Lutein: 0.37 Zeaxanthin: 0.09 for 3.2 y	889	Plasma β-cryptoxanthin and zeaxanthin were associated with reduced risk of gastric adenoma: 0.53 (0.30-0.94), P_{trend}=0.006 and 0.39 (0.22-0.69), P_{trend}=0.005.	α-carotene: ≥0.17 vs. <0.06 β-carotene: ≥0.49 vs. <0.22 Lycopene: ≥0.83 vs. <0.33 β-cryptoxanthin: ≥0.34 vs. <0.10 Lutein: ≥0.51 vs. <0.26 Zeaxanthin: ≥0.12 vs. <0.06	(Jenab et al. 2006)
Pooled	Meta-analysis	α- and β-carotene Lycopene β-cryptoxanthin Lutein/zeaxanthin for 4-25 y	1953-26300	Serum carotenoids may not protect from the risk of lung cancer.	Highest vs. lowest	(Gallicchio et al. 2008)
Japanese	Nested case-control	α- and β-carotene: 0.05 carotene: 0.05 Lycopene: 0.21 β-cryptoxanthin: 0.28 Lutein+zeaxanthin: 0.77 for 14y	1022	Plasma β-carotene was inversely associated with the risk of gastric cancer: 0.46 (0.28-0.75), P_{trend} <0.01.	α-carotene: >0.09 vs. <0.02 β-carotene: >0.72 vs. <0.08 Lycopene: >0.36 vs. 0.0 β-cryptoxanthin: >0.64 vs. <0.06 Lutein+zeaxanthin: >1.23 vs. <0.40	(Persson et al. 2008)

Table 10. Summary of epidemiological studies of the relation between carotenoid supplementation and risk of various cancers.

Study population	Type of study	Supplementation and duration	N	Effect on cancer RR/OR (95% CI), P-value	Comparison	Reference
Finnish men ATBC study[a]	Randomized, double-blind placebo-controlled trial	50 mg/d α-tocopherol alone or 20 mg/d β-carotene alone or both for 5-8 y	29133	β-carotene increased lung cancer risk: 1.16 (1.02-1.33), P=0.02 No effect on other cancers.	Highest quartile vs. lowest	(Albanes et al. 1996)
American CARET study[b]	Randomized, double-blind placebo-controlled trial	Combination of 30 mg/d of β-carotene and 25000 IU/d of retinol for 4 y	18314	Incidence and mortality of lung cancer increased. Risk for lung cancer: 1.28 (1.04-1.57), P=0.02 Risk for lung cancer mortality: 1.46 (1.07-2.00)	β-carotene group vs. placebo	(Omenn et al. 1996)
French SUVIMAX study[c]	Double-blind, placebo-controlled primary prevention trial	Combination of 120 mg vitamin C 30 mg vitamin E 6 mg β-carotene for 7.5 y	13017	Total cancer risk decreased in men, but not in women. β-carot: 0.59 (0.37-0.95), P=0.0285	Median	(Galan et al. 2005)
Washington state VITAL study[d]	Supplement questionnaire	β-carotene Lutein Lycopene, retinol for 10 y	77126	Risk of lung cancer increased. Lutein: 2.02 (1.28-3.17) β-carotene: 3.22 (1.29-8.07) Other were not associated with cancer	Lutein: ≥ 10 y use vs. nonuse β-carot: ≥ 4 y use vs. nonuse	(Satia et al. 2009)
Pooled	Meta-analysis	β-carotene 6-15 or 20-30 mg/d	1621-180702	No effect on incidence of all cancers, pancreatic, colorectal, prostate, breast cancers, melanoma and non melanoma. Incidence of lung and stomach cancers increased.	β-carotene group vs. placebo	(Druesne-Pecollo et al. 2010)

[a]Alpha-tocopherol, beta-carotene cancer prevention study, [b]The Beta-Carotene and Retinol Efficacy Trial, [c]The Supplementation en Vitamines et Mineraux Antioxydants
[d]The VITamins And Lifestyle study, N = number of subjects

exposure to indoor light, but not outdoor light, before processing or replacing air in vials with nitrogen gas did not alter concentrations substantially (Clark et al. 2004), suggesting that normal processing methods are acceptable for carotenoids.

Serum and plasma carotenoid concentrations were reported to be stable at -20°C for 5-9 months. Comstock et al. reported that α-tocopherol and carotenoids were all stable at -77°C for 4 years in plasma (Comstock et al. 1993). Thomas et al. found no evidence of degradation of retinol, α-tocopherol, and β-carotene in lyophilized serum, when stored at -80°C over the 10-year period (Thomas et al. 1998). By contrast, it was found that carotenoids, γ-tocopherol and α-tocopherol in plasma stored at -80°C were stable only for up to 8 months (Peng et al. 1994).

It has been observed that carotenoids become unstable after being extracted from plasma into organic solvents, particularly on exposure to light and heat. The stability of carotenoids extracted from plasma into hexane containing 0.01% BHT was investigated at different temperatures (Su et al. 1999). Variability in the measurement of lutein/zeaxanthin, β-cryptoxanthin, trans-lycopene, α-carotene and β-carotene was greater at room temperature that at either +4°C or -20°C. Statistically significant variations were observed in the measured concentrations of lutein/zeaxanthin, α-carotene and β-carotene in samples kept cold. However, the concentration decrease was small and of little biological significance, particularly over the first 24 h. At room temperature, the variability of concentrations was greater than in samples kept cold. Thus, storage of extracts at room temperature is not recommended (Su et al. 2002; Su et al. 1999).

2.4.2 Chromatographic methods

Methods based on chromatography involve physical separation in which carotenoids are distributed between two phases (stationary and mobile phase). The stationary phase may be solid or liquid. The mobile phase may be liquid or gas. HPLC, which has a liquid mobile phase, is the most popular analytical method for measuring carotenoids from biological materials. Gas chromatography (GC) has been rarely used for analysis of carotenoids, because these compounds are labile to destruction and isomerization at high temperatures, and they are not very volatile (De Leenher et al. 2000).

2.4.2.1 Sample preparation for chromatography

Carotenoids are usually soluble in lipids or in non-polar solvents, except when they exist as complexes with proteins and sugars. The most common method for sample purification is liquid-liquid extraction. In these methods, blood plasma or serum is often first treated with a polar solvent (usually ethanol) to precipitate proteins and then fat-soluble vitamins (e.g., retinol and tocopherols), and the carotenoids are extracted with hexane or ethyl acetate. Diethyl ether is also used for extractions. An internal standard is often dissolved in the organic solvent used to precipitate proteins. It is also necessary to add antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) during the extraction

process to prevent degradation of the carotenoids (Su et al. 2002). Hexane is the organic solvent most commonly used for the extraction of carotenoids from biological fluids (Olmedilla-Alonso et al. 2005; Lee et al. 2003; Lyan et al. 2001; Talwar et al. 1998; Gueguen et al. 2002). Thurnham et al. (1988) extracted carotenoids from plasma with heptane (Hexane does not carry water from the serum and is easy to evaporate under a gentle stream of nitrogen or argon. The organic extracts of plasma or other biological samples are usually concentrated to a known volume of suitable solvent before chromatographic analysis. Solid-phase extraction is also used to purify carotenoids from biological fluids prior to chromatographic analysis. The sample is added into an activated mini-column containing the stationary phase (usually derivatized silicagel). Proteins and other sample components are washed away and the carotenoids are finally eluted from the column with a suitable solvent. Dueker et al. (1993) used a C_{18} silica-based sorbent for the separation of retinol from its aqueous matrix followed by removal of lipid contaminants with an aminopropyl silica-based sorbent. In 2004, Chatzimichalakis et al. (2004) published a method using cyclohexyl columns for purifying carotenoids from blood serum and urine. A fully automatic method was developed for the determination of carotenoids in serum. In that method, a sample was injected into an automatic solid phase extraction (SPE) system for cleanup and pre-concentration, and then on-line transferred to a reversed-phase analytical column (Chatzimichalakis et al. 2004).

2.4.2.2 High performance liquid chromatography

Carotenoids are separated by their partition coefficient between the solid phase of the column and the HPLC eluent. Most of the HPLC methods for carotenoids are isocratic, but the separation efficiency can be improved by using gradient elution. The gradient is often based on an organic solvent.

Normal-phase liquid-chromatography methods are rarely used for measuring the serum and plasma concentrations of carotenoids. In a normal-phase system, the stationary phase is more polar than the mobile phase. McGeachin et al. (1995) published a normal phase HPLC method employing a hexane: ethyl acetate mobile phase gradient for separating and quantifying vitamin A and E and carotenoids from serum (McGeachin & Bailey 1995). Khachik et al. (1997) analyzed carotenoids and their metabolites from human milk and serum by using a silica-based, nitrile-bonded column and a mobile phase consisting of hexane: dichloromethane:methanol:N,N-diisopropylethylamine (Khachik et al. 1997). However, this normal-phase system requires a large volume of organic solvent waste.

Reversed-phase separation phases have been commonly used for HPLC carotenoid analysis. For clinical purposes and large epidemiological studies using human plasma, reversed-phase HPLC with ultraviolet (UV) detection using octadecylsilyl (C_{18}) column is an optimal approach. It is quick, simple, relatively cheap, shows good recovery (90–105%), low limits of detection and has good

reproducibility (inter-assay coefficient of variation (CV) <10%). In these systems, the mobile phase is more polar than the stationary phase (Su et al. 2002). With reversed-phase HPLC, octylsilyl (C_8) and C_{18} columns have proved to be well suited for analysis of carotenoids (Su et al. 2002). Most of the published methods have used a C_{18} HPLC column (e.g., Olmedilla-Alonso et al. 2005; Lee et al. 2003; Lyan et al. 2001; Talwar et al. 1998; Gueguen et al. 2002; Nierenberg & Nann 1992; Ortega et al. 2004). HPLC methods based on C_8 stationary phases are not common (Huck et al. 2000). Triacontanylsilyl (C_{30}) bonded columns are a good choice for analysis of carotenoids as well as mixtures of their geometrical isomers (Schweigert et al. 2003, Rajendran et al. 2005).

UV and visible single wavelength absorbance detectors have been used for many years as components of HPLC systems for quantitation of carotenoids. UV absorbance detection allows sufficient sensitivity for many routine purposes. Coupling a diode array detector (DAD) to the HPLC allows for a continuous collection of spectrophotometric data during analysis (Su et al. 2002), which greatly aids in determining peak purity and identifying unknown compounds in some cases. In addition, fluorescence detectors (Lee et al. 2003), electrochemical detectors (Finckh et al. 1999, Hermans et al. 2005; Lee & Ong 2009) and mass spectrometry (Khachik et al. 1997; Wang et al. 2000; Kelm et al. 2001,; Andreoli et al. 2004) have been used with HPLC to analyze retinol, tocopherols, tocotrienols, carotenoids and their isomers from human blood.

2.4.2.3 Other methods based on chromatography

Open-column and thin-layer chromatography methods were originally used for analysis of carotenoids. Historically, much of the "carotene" data in tables of food composition has been obtained by measuring absorption at a specified wavelength and quantified against a β-carotene standard, or more commonly by open-column chromatography to separate carotenoid pigments, which are then quantified spectrophotometrically. Open-column and thin-layer chromatography methods require large amounts of sample and have difficulties with total recovery of the carotenoids from thin layer chromatography (TLC) plates. These methods, clearly offer poorer resolution and assay speed than more modern methods (Su et al. 2002).

Sander et al. (Sander et al. 1994) first reported the use of polymeric C_{30} stationary phases in capillary electrochromatography (CEC) for the separation of carotenoid isomers in foods and algaes. Isocratic CEC separations were carried out using a Hewlett-Packard 3D capillary electrophoresis instrument with vial pressurization modification. Absorbance detection at 450 nm was employed with acetone and a 1 mM borate buffer as the mobile phase. This method was able to separate from food samples lycopene isomers, β-carotene isomers, α-carotene isomers, lutein isomers, zeaxanthin isomers and β-cryptoxanthin isomers (Sander et al. 1994).

2.4.3 Other techniques

The new Optothermal Window (OW) method was developed for measuring lycopene in tomato products. This method appeared to be a reliable, quick analytical tool for determining the concentration of lycopene from a large variety of commercial tomato products without sample purification (Bicanic et al. 2004). Hornero-Méndez et al. (2001) published a method for determining red and yellow carotenoid pigments from paprika and red pepper oleoresins based on UV-visible spectrophotometry. Wavelengths of 472 and 508 nm were used for simultaneous quantification of these pigments from the acetone extract (Hornero-Mendez & Minguez-Mosquera 2001).

3 Aims of the study

The aims of the present study were:

1. To develop a quick, simple and sensitive method for analyzing plasma concentrations of retinol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene.

2. To investigate and follow the plasma levels of carotenoids and fat-soluble vitamins in Eastern Finnish men and women from the late 1980s to the beginning of the 2000s, and the seasonal variation in carotenoid levels.

3. To investigate the effects of astaxanthin supplementation on lipid oxidation in healthy men by using various lipid oxidation markers.

4. To identify the main determinants of serum LDL conjugated dienes (focus on carotenoids, retinol and α-tocopherol).

5. To study the association between serum concentration of lycopene and the risk of cancer among eastern Finnish, middle-aged men in the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) study.

4 Materials and methods

4.1 STUDY SUBJECTS AND DESIGN

4.1.1 HPLC method development (I)

The subjects used in the development of the carotenoid measurement method were male volunteers from the Laboratory of the Research Institute of Public Health of University of Kuopio. Part of the subjects used in comparing the method, were from Spain.

4.1.2 Astaxanthin Supplementation Study (ASTA, II)

The study subjects were healthy non-smoking male volunteers aged 19–33 years with no severe diseases or malabsorption. The study was a randomized double-blind trial based on comparison of two parallel identically sized groups; the intervention group received 4 mg of astaxanthin (Astaxin®, AstaReal AB, Sweden) capsules twice per day (N=20) and the placebo group two identical-looking placebo capsules (microcrystalline cellulose) (N=20).

4.1.3 Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study (III, IV)

The KIHD is an ongoing population-based, cohort study that was designed to investigate risk factors for cardiovascular and other degenerative disease in a sample of middle-aged men in the city of Kuopio, Finland and its rural communities. Baseline examinations of men aged 42–60 were carried out between 1984 and 1989. Of the 3235 eligible male subjects, 2682 (82.9%) participated in the study. Baseline examinations of women aged 53–73 years were carried out between March 1998 and February 2001. Of 1173 eligible female subjects, 920 (78.4%) participated in the study. After the baseline, 696 had died and 1031 had severe illness, migrated from the area, refused, or could not be contacted. Thus, 1241 men and 634 women aged 61-80 years participated in this 20-year follow-up visit over the years 2005-2008. For study III, samples for serum LDL conjugated dienes and plasma carotenoids were collected and analysed from a randomly selected subgroup of 349 subjects (124 women and 225 men) during February-October, 2007 and January-March, 2008.

Four-year reexaminations (i.e., the entry for study IV) for those examined in 1986-1989 were conducted between March 1991 and December 1993. Data for testing the association between serum concentrations of lycopene and the risk of cancer among Eastern Finnish middle-aged men were available for 997 men.

To assess possible changes in the serum levels of carotenoids over about 20 years in the Eastern Finnish population, serum/plasma carotenoid concentration were measured in the KIHD subjects. Concentrations were measured from men in the 4 y reexamination visit during the years 1991-1993 (N=1031), 11 y

reexamination during the years 1998-2001 (N=851) and in the so-called 20 y reexamination during the years 2005-2008 (N=1228), with the follow-up time ranging between 16-24 years. However, the 20 y visits were carried out at the same quarter of the year as the baseline examinations. In women, the concentrations were measured at the baseline visit during the years 1998-2001 (N=913) and at the 7 y reexamination during the years 2005-2008 (N=620).

4.1.4 Permission of the Ethics Committee

The Ethics Committee of the Northern Savo Hospital District accepted the substudies II-IV for this thesis.

4.2 Blood sampling (I, II, III, IV)

Subjects were instructed to abstain from eating and smoking for 12 hours (I, II, III, IV) and from consuming alcohol for 2 days (II, III) or 3 days (IV) before blood sampling. Fasting venous blood samples were collected into vacuum tubes (Terumo, Leuven, Belgium) without tourniquet after the subject had rested in the supine position for 5 min (I, II, III) or 30 min (IV). Blood samples were centrifuged at 1500 × g for 10 min after collection, and serum or plasma was separated immediately after centrifugation. Blood for carotenoids was collected in lithium heparin tubes (I, III) that were kept on ice until centrifugation and serum tubes (IV) and EDTA tubes (II). Frozen samples were stored at −70/-80°C until used. The blood cell profile was analysed from fresh EDTA blood (II, IV). Blood for the measurement of LDL conjugated dienes (III) was drawn into serum tubes, and samples were centrifuged at 1500 × g for 10 min within 30 min after blood collection. After separation of serum, 12 µl of 100 mg/ml EDTA was added to 1.2 ml of serum to avoid ex vivo oxidative modification of LDL and an analysis was performed immediately from the fresh sample.

4.3 Analysis of astaxanthin, other carotenoids and vitamins

For the original publication II, plasma astaxanthin and other carotenoids (lutein-zeaxanthin, canthaxanthin, lycopene, β-cryptoxanthin and β-carotene) were determined by Professor Anders Olsson in Uppsala, Sweden. In the original work IV, an HPLC method based on the method by Thurnham et al. (Thurnham et al. 1988) was used for the determination of retinol, α-tocopherol and β-carotene serum concentrations. This method lacks lutein-zeaxanthin, canthaxanthin and β-cryptoxanthin.

The HPLC method for determination of plasma carotenoids (lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene) and fat-soluble vitamins (retinol and α-tocopherol) developed in work I was also used in work III. Briefly, the frozen Li-heparin plasma samples were thawed at room temperature and 200 µl was pipetted into glass tubes. A volume of 500 µl of ethanol-0.01% (w/v) BHT containing α-tocopherol acetate and β-Apo-8'-carotenal as internal standards, was added. Thereafter, water and hexane were added and samples were extracted. The

samples were centrifuged at 1500 × g for 5 min at +4°C and frozen at -70°C for 30 min. The upper organic layer was decanted into a glass tube and evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried residue was reconstituted in 200 µl of the mobile phase into the HPLC. In work III, lipid standardized values of retinol and α-tocopherol (Jordan et al. 1995) were used for statistical analyses.

Ascorbic acid was analysed by high-performance liquid chromatography using the ion-exchange method (Parviainen et al. 1986) (II).

Serum folate was measured by using a radioimmunoassay (Quantaphase II; Bio-Rad, Hercules, CA) (Rissanen et al. 2001).

4.4 MEASUREMENT OF LIPID OXIDATION IN VITRO AND IN VIVO

4.4.1 LDL and VLDL to oxidation in vitro (II)

Combined VLDL and LDL were isolated from fresh ethylene diamine tetra-acetic acid (EDTA) plasma by density gradient ultracentrifugation (KBr gradient). Since EDTA blocks the lipoprotein reaction with Cu^{2+}, EDTA was removed from the LDL fraction by using small gel filtration PD-10 columns (Pharmacia, Uppsala, Sweden). Briefly, VLDL+LDL fraction was diluted with oxygen-saturated phosphate buffered saline and the formation of conjugated dienes was initiated by adding copper chloride to the diluted VLDL+LDL fraction and the reaction was followed by spectrophotometry at 234 nm (Kaikkonen et al. 1997; Nyyssönen et al. 1997). The lag time, and the maximum reaction velocity (V_{max}) were determined at +37°C by a temperature controlled Beckman Du 640i spectrophotometer with an enzyme kinetics data system (Beckman Co, Brea, CA, USA).

4.4.2 Plasma hydroxy fatty acids and free F_2-isoprostanes (II)

Plasma (Li-heparin) C_{18} hydroxy fatty acids (8, 9, 10, 11, 12, 13, 15 and 16-mono hydroxy fatty acids) (Wilson et al. 1997) as well as plasma free F_2-isoprostanes were measured using a gas chromatograph/mass spectrometer (Agilent Technologies, Espoo, Finland) (Morrow et al. 1999).

4.4.3 Serum LDL conjugated dienes (III)

The content of conjugated dienes in precipitated LDL was determined spectrophotometrically. Briefly, serum LDL was precipitated with buffered heparin, and resuspended in 0.1 M phosphate buffered salin, pH 8.0. The cholesterol concentration was determined, and the rest of the suspension was used for measuring conjugated dienes. Lipids were extracted from the LDL with a mixture of chloroform: methanol (3:1), evaporated to dryness with a gentle stream of nitrogen, and reconstituted in cyclohexane. The concentrations of conjugated dienes were measured spectrophotometrically at 234 and 300 nm.

4.4.4 Other biochemical measurements (II, III, IV)

Serum paraoxonase (PON) activity was measured based on its capacity to hydrolyze paraoxonase on a microtiter plate (Thermo Electron Oy, Vantaa, Finland) (Mackness et al. 1991). Uric acid was measured using an enzymatic colorimetric method (Randox Laboratories Ltd, UK).

Concentrations of serum interleukin 6 and interleukin 2-receptor were analysed by a solid-phase Enzyme Amplified Immunoassay on a microtiter plate (BioSource Europe SA, Nivelles, Belgium). Plasma C-reactive protein (CRP) was determined with a high sensitivity particle-enhanced immunoturbidimetric assay (CRP Latex HS, Roche/Hitachi 911, Roche Diagnostics GmbH, Mannheim, Germany).

Concentrations of serum total cholesterol (Konelab 20XT, Thermo Fisher Scientific, Vantaa, Finland) and triglycerides (Roche Diagnostics, Mannheim, Germany) were analyzed with enzymatic methods. Serum HDL cholesterol was measured from the supernatant after magnesium chloride dextran sulfate precipitation with enzymatic method (Thermo Fisher Scientific), and the enzyme activity of serum gamma-glutamyl transferase (γ-GT) with the method recommended by International Federation of Clinical Chemistry and Laboratory Medicine (Thermo Fisher Scientific). Blood glucose was measured using a hexokinase method (Thermo Fisher Scientific) after precipitation proteins by trichloroacetic acid. Leukocytes were analyzed by Advia 60 blood cell counter (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

Serum fatty acids were analyzed after chloroform-methanol extraction and methylation with sulphuric acid–methanol. The methylated fatty acids were analyzed by a gas chromatograph equipped with a flame ionization detector.

4.4.5 Assessment of nutrient intake (II)

The consumption of foods was assessed at the time of blood sampling during the baseline phase and at the end of this study. Subjects were instructed on the use of household measures for quantitative recording of their food intake during the 4-day data collection. A nutritionist gave instructions and checked the completed food intake records. Dietary intake of nutrients and foods was calculated using NUTRICA software (version 2.5; The Social Insurance Institution of Finland, Turku, Finland). This software is compiled using mainly Finnish values for the nutrient composition of foods, and takes into account the losses of vitamins in food preparation. In total, the database contains comprehensive data for 1300 food items and dishes, as well as 30 nutrients (Rissanen et al. 2003).

4.4.6 Other measurements (II, III, IV)

The waist-to-hip ratio was defined as the circumference of the waist girth/hip measured at the trochanter major. The body mass index (BMI) was computed as the ratio of weight (kilograms) to the square of height (meters). Alcohol ingestion was assessed with a questionnaire structured quantity-frequency method on

drinking behaviour over the previous 12 months. Education was coded into three categories based on years of education (<6, 7-11, and 12 or more years). Family history of cancer was asked by a self-administered questionnaire and checked by the interviewer (Laukkanen et al. 2004). Physical activity was assessed using a 12-month leisure-time history based on self-reported information about frequency per month over the preceding year, average duration per occasion, and intensity level. Metabolic units were assigned for each activity according to intensity (Salonen et al. 1991). Physical activity was expressed as kcal/d. Current smoking was asked before blood drawing, and the lifelong exposure to smoking was based on smoking years. Resting blood pressure was measured in the morning by a trained nurse with a random-zero mercury sphygmomanometer (Hawksley, Lancing, United Kingdom). Symptomatic ischaemic heart disease (IHD) or IHD history and medications were asked by a self-administered questionnaire, and checked by the interviewer.

4.5 Statistical analyses

Paired samples t-test (P <0.05) was used to statistically assess the differences of concentrations of retinol, α-tocopherol and carotenoids between the compared HPLC methods (I). A correlation between two HPLC methods was tested both before and after logarithmic transformation of carotenoid concentration distribution.

In the ASTA study (II), differences between baseline and three-month values with the pooled groups were tested by the paired samples t-test. Means were compared across the study groups by the independent samples t-test.

In work III, normal distributions of variables were tested by the Kolmogorov-Smirnov test, and biased distributions of lipid standardized retinol and α-tocopherol and plasma carotenoids were logarithmically transformed before association analyses. Before logarithmic transformation, one was added to carotenoid concentration [log(1+x)] to avoid negative values. Associations between serum LDL conjugated dienes and the other measurements were estimated by Pearson's correlation coefficients. Multivariate regression analysis was used to identify the determinants of serum LDL conjugated dienes. Each of the fat-soluble vitamins (α-tocopherol and retinol) or carotenoids (lycopene, α-carotene and β-carotene, lutein, zeaxanthin and β-cryptoxanthin) was tested separately in multivariate-regression analyses together with other possible determinants.

In the KIHD cancer follow-up study (IV), descriptive data were presented as mean and standard deviation. Means between cancer cases and the other men were compared by analysis of variance (ANOVA). RRs and 95% CIs for cancer in tertiles of the serum concentrations of lycopene were estimated by using the Cox proportional hazard model. Tests for linear trends were performed by ANOVA. Correlations between the levels of serum lycopene, other serum antioxidants and years of smoking were assessed using Pearson's correlation coefficients. Changes in

the serum/plasma levels of carotenoids over the 20-year period within the Eastern Finnish population were tested using ANOVA.

5 Results

5.1 MEASUREMENT OF CAROTENOIDS AND METHOD VALIDATION (I)

5.1.1 Optimization of sample preparation

In original work I, it was clearly observed that after single extraction, the addition of ultrapure water enabled the aqueous layer below the hexane phase to freeze, thus accelerating phase separation without pipetting. The developed method appeared to be less tedious and time-consuming than traditional extraction methods, requiring removal of the organic layer by pipetting. The freezing method shortened the time spent for sample handling, recovered the hexane layer completely, and significantly improved the repeatability of the results. The extracts were also cleaner, because the frozen aqueous layer prevents mixing of the layers during separation. Table 11 compares the data for intra-assay repeatability, when organic layer was removed using the traditional method and when removed by decanting after freezing.

Table 11. Repeatability of plasma retinol, α-tocopherol and carotenoids by using traditional extraction method and freezing method. Intra-assay CVs were determined with the pooled Li-heparin plasma of several volunteers.

	Pipetting		**Decanting**		
	Intra-assay[a] (N=10)		**Intra-assay[b] (N=10)**		
Compound	**Mean ± SD (µmol/l)**	**CV%**	**Mean ± SD (µmol/l)**	**CV%**	***P*-value**
Retinol	2.30 ± 0.05	2.0	2.47 ± 0.04	1.7	
α-Tocopherol	27.4 ± 0.36	1.3	31.7 ± 0.33	1.1	
Lutein	0.27 ± 0.01	2.5	0.26 ± 0.01	2.6	
Zeaxanthin	0.059 ± 0.005	8.8	0.065 ± 0.003	4.0	
β-Cryptoxanthin	0.33 ± 0.014	4.3	0.27 ± 0.004	1.7	
Lycopene	0.30 ± 0.011	3.8	0.24 ± 0 .003	1.3	
α-Carotene	0.19 ± 0.006	3.2	0.17 ± 0.003	2.1	
β-Carotene	0.41 ± 0.01	3.5	0.60 ± 0.01	1.7	
	Mean	3.7	**Mean**	2.0	0.025

[a]Hexane layer (5 ml) was separated by pipetting, [b]Hexane layer (2 ml) was separated by decanting after freezing at -70ºC, SD = standard deviation, N = Number

5.1.2 Linearity, recovery and precision

Results are from original publication I. The linearities and limits of detection (LODs) of the method for fat-soluble vitamins (retinol and α-tocopherol) and carotenoids were determined by analysing a series of diluted pure standard

mixture. The linearities for all compounds were excellent (r >0.999). The LODs were 0.010–0.73 μmol/l exceeding signal-to-noise ratio of 3:1. Concentrations above the LOD values were regarded as quantifiable, although the variation was higher than determined for the method with the quality control samples. Spiked plasma samples at three concentrations with triplicate measurements were analysed to test the efficiency of the extraction procedure. The absolute and relative recoveries of all compounds ranged from 80 to 103%. The intra-assay and inter-assay CVs were determined by analysing the frozen Li-heparin plasma pool and Euro-Trol quality controls (QCs). The intra-assay CVs of the pooled Li-heparin plasma ranged from 1.1 to 4.0% (normal level) and 3.3 to 9.0% (low level) (N=10). The inter-assay CVs were obtained by analysing plasma pool during the years 2006-2009 (N=162). CVs were 5.6–8.7%. The inter-assay CVs of Euro-Trol QCs were obtained by analysis during the years 2006-2009. The inter-assay CVs were 7.6–20.6% (N=114) and 6.7-11.2 (N=17) (Level 1). At level 3, CVs were 7.3-11.7 (N=119) and 5.4-10.7 (N=17). The results for each compound are documented in original publication I.

5.1.3 Stability of extracted sample

Results are decscribed in original publication I. In order to determine how long storage time affects the concentrations of fat-soluble vitamins and carotenoids, the extracted plasma pool was stored on the autosampler at +4°C and analysed every day for one week. We observed that the concentration of retinol increased during the one-week storage up to 16%, which indicated that the solvent may have evaporated into the vial and that the retinol molecule was stable during the storage period. The peak height of the internal standard decreased 7%, indicating that it had degraded during the one-week period. The increase in the concentration of retinol was not compensated for by the internal standard. After two days, the mean concentrations of lycopene and β-carotene decreased by 13% and 11%, respectively. After a week, these concentrations decreased even further being 29% and 23% lower than just after extraction, respectively. However, lycopene and β-carotene decomposed even more than shown by the percentage values, due to the opposite effect of solvent evaporation. A slight decreasing trend was seen in the concentrations of α-tocopherol and other carotenoids. These results suggest that extracted samples can be stored for no more than three days at +4°C without a significant decrease in the concentrations of the carotenoids, except for lycopene and β-carotene, which seemed to be the most sensitive carotenoids for degradation.

5.1.4 Method comparison

For comparing the methods, 10 plasma samples from Kuopio and 24 serum samples from Madrid were measured using the HPLC methods at the University of Kuopio (Finland) and the Hospital Universitario Puerta de Hierro (Madrid, Spain). The correlation coefficients (r) between the two HPLC methods were acceptable both without (original publication I) and with logarithmic transformation (Table 12) of the carotenoids and fat-soluble vitamin

concentrations. For zeaxanthin, no correlation was found between Kuopio and Madrid. The percentage differences ranged from 0.30% to 26% for the carotenoids, except for zeaxanthin. The concentrations of retinol, α-tocopherol, zeaxanthin, β-cryptoxanthin, lycopene and α-carotene differed significantly between the two HPLC methods. However, the percentage difference for retinol (-5.9%) and α-carotene (-9.8%) were considered to be acceptable (Table 12). These methods were also compared with Bland-Altman bias plots, which present the percentage difference between the two methods. On average, the measurement levels of retinol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin and lycopene were higher in Madrid than in Kuopio. For retinol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin and lycopene the difference between the methods was independent of the analyte concentration in the sample, whereas for α-carotene and β-carotene the difference was higher at the low levels of the carotenoids (broader scattering at low concentration (Figure 5).

Table 12. Concentrations of retinol, α-tocopherol and carotenoids in adult volunteers measured by two HPLC methods.

Compound	Kuopio (µmol/l)	Madrid (µmol/l)	Correlation coefficient (r) Logaritmic scale	Difference %	P value
Retinol	1.83 ± 0.58	1.95 ± 0.59	0.9509	-5.9	<0.0001
α-Tocopherol	28.8 ± 5.4	24.6 ± 4.8	0.8442	17.1	<0.0001
Lutein	0.24 ± 0.10	0.24 ± 0.08	0.8702	-0.3	0.750
Zeaxanthin	0.050 ± 0.02	0.070 ± 0.04	0.0005	-33.9	0.001
β-Cryptoxanthin	0.20 ± 0.14	0.25 ± 0.18	0.9852	-20.4	<0.0001
Lycopene	0.23 ± 0.07	0.31 ± 0.11	0.8071	-26.2	<0.0001
α-Carotene	0.11 ± 0.10	0.12 ± 0.11	0.9536	-9.8	0.012
β-Carotene	0.40 ± 0.28	0.40 ± 0.31	0.9836	-0.5	0.517

All values are mean ± SD (N = 34), SD = standard deviation

5.1.5 Concentrations of retinol, α-tocopherol and carotenoids in the KIHD study population within 20-year follow-up

Results are previously unpublished, based on the populations described in publications III and IV. In both genders, the concentration of lycopene decreased significantly between follow-up years 4–20 and 11–20 (P<0.0001) (Table 13). Except for retinol, α-tocopherol, α-carotene (P<0.0001) and β-carotene (P=0.006) concentrations differed significantly in men between the follow-up years. In women, the concentrations tended to increase (P<0.0001). Women also had significantly higher concentrations of α-tocopherol and carotenoids than men, except for lycopene. Lycopene concentrations were at the same level for both genders.

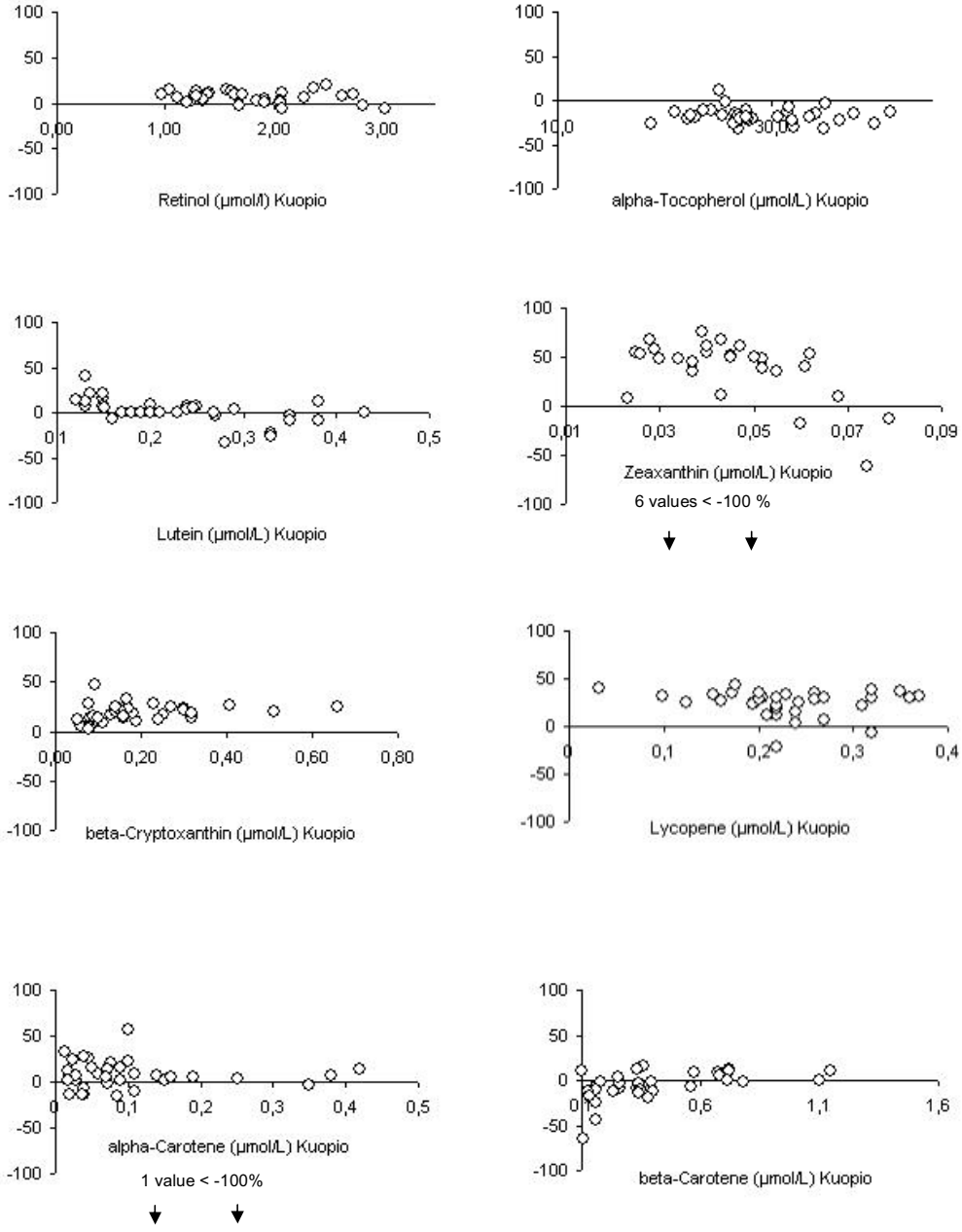

Figure 5. Bland-Altman bias plots of the concentrations of carotenoids between two HPLC methods. X-axis = concentration in Kuopio. Y- axis = difference between two HPLC methods (Madrid-Kuopio) (%).

Table 13. Mean concentrations and standard deviations of serum/plasma retinol, α-tocopherol and carotenoids in the KIHD study population in men and women between follow-up years (4, 11 and 20 y).

	Men (n=2569-560)				Women (n=615)	
Compound	y 1984-89 (µmol/l) Mean±SD)	4 y (µmol/l) (Mean±SD)	11 y (µmol/l) (Mean±SD)	20 y (µmol/l) (Mean±SD)	y 1998-2001 (µmol/l) (Mean±SD)	7 y (µmol/l) (Mean±SD)
Retinol	2.11 ± 0.48	2.12 ± 0.43	1.92 ± 0.51	2.11 ± 0.52	1.73 ± 0.45	2.07 ± 0.58
α-Tocopherol	20.3 ± 5.8	28.5 ± 7.2	28.7 ± 8.1	32.7 ± 7.6	29.4 ± 7.1	36.3 ± 8.0
Lycopene	ND	0.17 ± 0.14	0.22 ± 0.18	0.10 ± 0.08	0.23 ± 0.17	0.11 ± 0.07
α-Carotene	ND	0.10 ± 0.08	ND	0.12 ± 0.10	ND	0.17 ± 0.13
β-Carotene	ND	0.40 ± 0.27	0.37 ± 0.25	0.43 ± 0.30	0.55 ± 0.40	0.62 ± 0.40
Lutein	ND	ND	ND	0.24 ± 0.09	ND	0.26 ± 0.11
Zeaxanthin	ND	ND	ND	0.038 ± 0.019	ND	0.040 ± 0.017
β-Cryptoxanthin	ND	ND	ND	0.11 ± 0.10	ND	0.16 ± 0.17

ND = not determined, SD = standard deviation

In both men and women, the concentrations of serum lycopene and β-carotene significantly differed between different seasons (Table 14). The concentration of serum lycopene was at the lowest in late autumn and early winter. The serum β-carotene concentration was highest in the late summer and autumn, and β-carotene levels were also high in the winter of 2005. During the 20 y follow-up, plasma lutein and zeaxanthin concentrations were the lowest in the late autumn and early winter. The concentration of plasma β-cryptoxanthin was highest in midwinter and early spring.

At baseline and 11-year visits, the subjects kept a 4-day food diary for collecting nutrient intake. Correlations between the energy-adjusted dietary intake and serum concentrations of lycopene were 0.34 for men and women (n=940), 0.33 for women (N=499) and 0.35 for men (N=441), In addition, for β-carotene, the correlations were 0.36 (N=1717), 0.34 (N=900) and 0.38 (N=817), respectively.

5.2 EFFECT OF CAROTENOIDS ON OXIDATIVE MODIFICATION OF LIPIDS (II, III)

5.2.1 Astaxanthin Supplementation Study (II)

Healthy young men received 8 mg/d of astaxanthin supplement for 3 months. The concentrations of plasma 12- and 15-hydroxy fatty acids significantly declined (by 36% and 60%, respectively) in the astaxanthin group (P=0.048 and P=0.047, respectively) during supplementation, but not in the placebo group.

No difference was observed in the concentrations of 12-hydroxy fatty acids between placebo and astaxanthin treated groups at 3 months. The concentration of plasma 15-hydroxy fatty acid decreased by 22%, though the difference between the astaxanthin and placebo groups was not significant (P=0.056) No other statistically

Table 14. Mean concentrations and standard deviations of serum/plasma retinol, α-tocopherol and carotenoids in the KIHD study population in men and women between seasons and follow-up years (4, 11 and 20 y).

	Quartile of season				
			MEN		
Compound	1-3[a] (µmol/l) (Mean±SD)	4-6[b] (µmol/l) (Mean±SD)	7-9[c] (µmol/l) (Mean±SD)	10-12[d] (µmol/l) (Mean±SD)	*P* for difference between seasons[e]
			years 1991-93, 4 y (n=1031)		
Retinol	2.12 ± 0.45	2.07 ± 0.47	2.15 ± 0.46	2.10 ± 0.44	0.217
α-Tocopherol	29.3 ± 7.6	28.8 ± 7.6	28.9 ± 9.2	27.8 ± 7.5	0.200
Lycopene	0.15 ± 0.14	0.17 ± 0.14	0.17 ± 0.15	0.12 ± 0.11	<0.0001
α-Carotene	0.10 ± 0.07	0.09 ± 0.09	0.10 ± 0.07	0.10 ± 0.08	0.701
β-Carotene	0.35 ± 0.26	0.40 ± 0.39	0.43 ± 0.28	0.35 ± 0.21	0.013
			years 1998-2001, 11 y (n=851)		
Retinol	1.99 ± 0.59	1.94 ± 0.60	1.90 ± 0.48	1.92 ± 0.62	0.419
α-Tocopherol	28.6 ± 8.4	29.0 ± 7.7	28.7 ± 8.6	29.4 ± 8.0	0.777
Lycopene	0.16 ± 0.15	0.19 ± 0.17	0.23 ± 0.18	0.24 ± 0.20	<0.0001
α-Carotene	ND	ND	ND	ND	
β-Carotene	0.31 ± 0.20	0.34 ± 0.26	0.38 ± 0.23	0.44 ± 0.33	<0.0001
			years 2005-8, 20 y (n=1228)		
Retinol	2.12 ± 0.46	2.21 ± 0.55	2.20 ± 0.55	2.09 ± 0.54	0.006
α-Tocopherol	32.4 ± 7.3	34.6 ± 8.4	32.7 ± 7.8	31.9 ± 7.0	<0.0001
Lycopene	0.08 ± 0.07	0.09 ± 0.07	0.11 ± 0.08	0.08 ± 0.07	<0.0001
α-Carotene	0.13 ± 0.12	0.10 ± 0.07	0.10 ± 0.07	0.14 ± 0.13	<0.0001
β-Carotene	0.47 ± 0.38	0.41 ± 0.29	0.44 ± 0.27	0.47 ± 0.33	0.032
Lutein	0.24 ± 0.08	0.25 ± 0.10	0.25 ± 0.10	0.22 ± 0.08	0.0004
Zeaxanthin	0.038 ± 0.017	0.039 ± 0.019	0.041 ± 0.021	0.035 ± 0.017	0.0006
β-Cryptoxanthin	0.14 ± 0.10	0.12 ± 0.11	0.07 ± 0.07	0.09 ± 0.09	<0.0001
			WOMEN		
			years 1998-2001, Baseline (n=913)		
Retinol	1.72 ± 0.50	1.71 ± 0.43	1.81 ± 0.47	1.72 ± 0.44	0.056
α-Tocopherol	28.9 ± 6.9	30.6 ± 6.7	30.8 ± 7.1	28.3 ± 7.4	0.0001
Lycopene	0.17 ± 0.15	0.20 ± 0.16	0.26 ± 0.20	0.22 ± 0.16	<0.0001
α-Carotene	ND	ND	ND	ND	
β-Carotene	0.51 ± 0.38	0.46 ± 0.34	0.52 ± 0.34	0.59 ± 0.43	0.003
			years 2005-8, 7 y (n=620)		
Retinol	2.11 ± 0.63	2.06 ± 0.53	2.13 ± 0.59	2.00 ± 0.56	0.178
α-Tocopherol	36.5 ± 8.6	37.7 ± 7.7	35.9 ± 7.4	35.1 ± 8.2	0.025
Lycopene	0.09 ± 0.06	0.11 ± 0.08	0.13 ± 0.07	0.10 ± 0.07	0.0001
α-Carotene	0.19 ± 0.13	0.14 ± 0.12	0.15 ± 0.11	0.20 ± 0.14	<0.0001
β-Carotene	0.69 ± 0.47	0.55 ± 0.39	0.60 ± 0.33	0.64 ± 0.37	0.016
Lutein	0.25 ± 0.10	0.27 ± 0.10	0.29 ± 0.13	0.24 ± 0.09	0.002
Zeaxanthin	0.042 ± 0.021	0.042 ± 0.017	0.041 ± 0.018	0.036 ± 0.014	0.010
β-Cryptoxanthin	0.20 ± 0.18	0.15 ± 0.13	0.11 ± 0.08	0.16 ± 0.22	<0.0001

[a]Quartile 1-3: January-March, [b]Quartile 4-6: April-June, [c]Quartile 7-9: July-September,
[d]Quartile 10-12: October-December,[e]ANOVA, ND = not determined; SD = standard deviation

significant differences were found between the study groups in serum lipids, copper-induced lipid peroxidation, plasma free F_2 isoprostanes or serum fatty acid profiles.

Astaxanthin was well tolerated during the 3-month supplementation period. There was no gastrointestinal tract distress or any other side effects. The changes in liver enzymes did not differ significantly between astaxanthin and placebo groups. Neither the changes in basic blood profile nor changes in blood pressure were statistically significant between the study groups.

5.2.2 The Kuopio Ischaemic Heart Disease Risk Factor (KIHD) study (III)

In a statistical preanalysis, we found that gender was an important determinant of LDL conjugated dienes. Thus, in work III, men and women were analysed separately. Main determinants of serum LDL conjugated dienes in women (N=124) and men (N=225) are described in Table 15.

In the linear regression analyses, plasma lycopene concentration (standardized β=–0.33; *P*=0.002) was the strongest determinant of serum LDL conjugated dienes in women. Lycopene explained 7.9% of the variation in conjugated dienes.

Plasma β-carotene (standardized β=–0.23; *P*=0.002) appeared to be the most important determinant of conjugated dienes in men, accounting for 4.8% of the variation. The regression model with lycopene contributed to 29% of the variation in serum LDL conjugated dienes for the women, and the model with β-carotene to 15% of the variation in serum LDL conjugated dienes for the men. In women, apart from lycopene, lutein was inversely related with the dienes in explaining 4.1% of the variation (standardized β=–0.22; *P*=0.027).

All regression models showed significant inverse associations between diastolic blood pressure and conjugated dienes in women, but not in men. The use of statin medication was associated with increased LDL conjugated diene concentrations in both sexes. In men, age was associated with conjugated dienes in all and triglycerides in almost all statistical models independent of the antioxidant included. Neither lipid-standardized retinol nor α-tocopherol was associated with the conjugated dienes in men or women.

5.3 SERUM LYCOPENE AND THE RISK OF CANCER (IV)

In original work IV, a total of 141 cancers occurred during an average follow-up of 12.6 years, of which 55 cases were prostate cancers, 17 lung cancers, 16 intestinal cancers, 10 urinary bladder cancers, and the remaining were of other origin (e.g., lymphomas, gastric, oral cavity, liver, renal, cerebral, skin and pancreatic cancers).

The mean serum lycopene concentration was 0.12 μmol/l in the subjects with cancer and 0.16 μmol/l in the subjects without cancer during the follow-up. The RR in the highest tertile of the serum concentrations of lycopene was 0.63 (95% CI, 0.40-0.98; *P*=0.041) as compared to the lowest tertile, after adjusting for age and examination years (Figure 6). After further adjustment for family history of cancer, waist-to-hip ratio, years of smoking, intake of alcohol, education, physical activity

Table 15. Main determinants of serum LDL conjugated dienes in women (N=124) and men (N=225).

	Lipid standardized α-tocopherol				Lipid standardized retinol			
	α-Tocopherol				Retinol			
	Women		Men		Women		Men	
	β	*P*	β	*P*	β*	P	β	*P*
Log antioxidant	-0.103	0.282	-0.132	0.070	0.053	0.597	-0.026	0.716
Age	-0.015	0.878	0.174	0.017	-0.006	0.948	0.177	0.016
BMI	0.118	0.260	0.070	0.344	0.121	0.255	0.076	0.310
DBP	-0.251	0.014	0.009	0.902	-0.248	0.016	0.013	0.855
Statin medication	0.225	0.026	0.151	0.062	0.200	0.049	0.143	0.078
TG	0.015	0.883	0.176	0.020	0.019	0.852	0.145	0.053
	Lycopene				**α-Carotene**			
	Women		Men		Women		Men	
	β	*P*	β	*P*	β*	*P*	β	*P*
Log antioxidant	-0.328	0.002	-0.047	0.528	-0.213	0.050	-0.109	0.132
Age	-0.120	0.237	0.171	0.021	-0.051	0.614	0.178	0.014
BMI	0.098	0.323	0.073	0.330	0.062	0.568	0.053	0.482
DBP	-0.222	0.023	0.023	0.750	-0272	0.007	0.017	0.810
Statin medication	0.176	0.065	0.144	0.076	0.180	0.070	0.136	0.092
TG	-0.039	0.687	0.147	0.049	-0.018	0.855	0.141	0.058
	β-Carotene				**Lutein**			
	Women		Men		Women		Men	
	β	*P*	β	*P*	β*	*P*	β	*P*
Log antioxidant	-0.208	0.073	-0.233	0.002	-0.224	0.027	-0.023	0.748
Age	-0.017	0.859	0.185	0.009	-0.030	0.758	0.178	0.015
BMI	0.076	0.475	0.016	0.830	0.068	0.521	0.072	0.340
DBP	-0246	0.014	0.016	0.830	-0.259	0.009	0.072	0.340
Statin medication	0.193	0.051	0.149	0.059	0.202	0.038	0.078	0.281
TG	-0.056	0.604	0.131	0.073	0.002	0.984	0.149	0.047
	Zeaxanthin				**β-Cryptoxanthin**			
	Women		Men		Women		Men	
	β	*P*	β	*P*	β*	*P*	β	*P*
Log antioxidant	-0.151	0.127	-0.095	0.185	-0.053	0.598	-0.123	0.088
Age	-0.017	0.864	0.177	0.015	-0.013	0.898	0.182	0.012
BMI	0.119	0.252	0.068	0.367	0.128	0.224	0.081	0.276
DBP	-0265	0.009	0.012	0.869	-0.260	0.011	0.016	0.819
Statin medication	0.199	0.044	0.140	0.083	0.207	0.039	0.168	0.041
TG	0.001	0.989	0.160	0.033	0.015	0.881	0.160	0.032

Antioxidant = lipid standardized α-tocopherol or retinol, lycopene, α-carotene, β-carotene, lutein, zeaxanthin or β-cryptoxanthin, in turn. β = standardized regression coefficient, Statin medication, 0 = no, 1 = yes, BMI = Body mass index, DBP = Diastolic blood pressure TG = triglycerides. Blood leucocyte count was included in all the models

and serum folate, men in the highest tertile of the serum concentrations of lycopene had a 45% lower risk of cancer than those in the lowest serum lycopene tertile (RR=0.55; 95% CI, 0.34-0.89; *P*=0.015). With serum lycopene treated as a continuous variable, the adjusted RR was 0.18 (95% CI, 0.038-0.86; *P*=0.031). Serum retinol, α-tocopherol, α-carotene and β-carotene were not related to a risk of cancer when

examined as continuous variable. No association was observed between lycopene and prostate cancer risk in our cohort. For other cancers, after adjustment for covariates, men in the highest tertile of serum concentrations of lycopene had a 57% lower risk of other cancers compared with the lowest serum lycopene tertile.

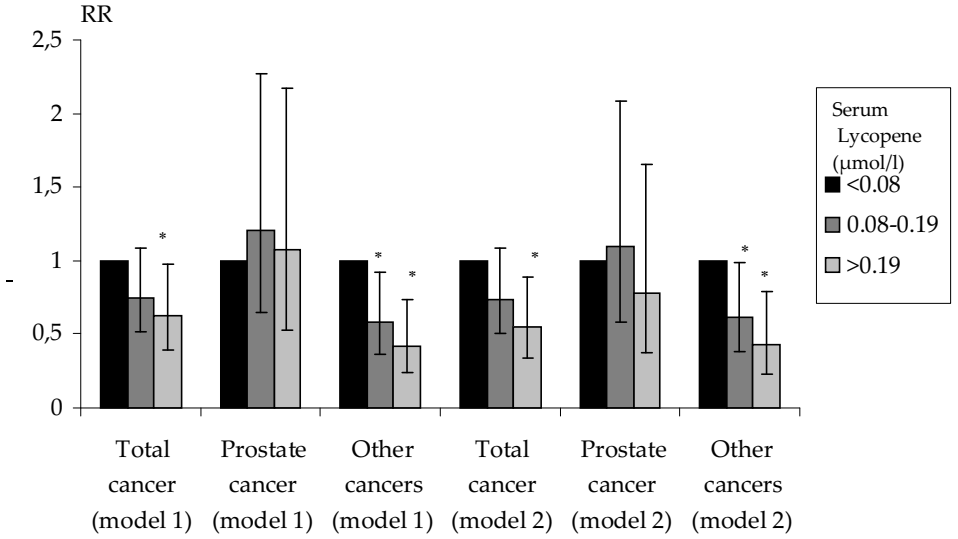

Figure 6. Relative risks (RR) and 95% confidence intervals (CI) of cancers by tertiles of serum levels of lycopene by using Cox proportional hazard's model after adjustment for age and examination year (model 1); model 1 + family history of cancer, waist-to-hip ratio, years of smoking, intake of alcohol, education, physical activity and serum folate (model 2).
** P <0.05.*

6 Discussion

6.1 METHODOLOGY

Liquid-liquid extraction with hexane is the most often used method for purification of carotenoids from biological fluids, since carotenoids are usually soluble in non-polar solvents (Su et al. 2002). At a preliminary stage in the present work, the sample preparation process included precipitation of proteins with 1 ml of ethanol containing internal standards and thereafter single extraction with 5 ml of hexane (Porkkala-Sarataho et al. 1996). Hexane layer separation turned out to be laborious and time-consuming, since separation was carried out by pipetting. A more convenient, faster sample preparation would provide for a larger number of samples to be prepared in a working day, a highly desirable feature in large

studies. A critical step in the liquid-liquid extraction is the phase separation. We clearly observed that after single extraction, the addition of ultrapure water enabled the aqueous layer below the hexane phase to freeze. The present method offers five advantages: 1) freezing of the aqueous layer speeds up the phase separation step; 2) the developed method appears to be less tedious than traditional extraction methods, which require removal of organic layer by pipetting; 3) the freezing method shortens the time spent in sample handling; 4) our method allows a more complete recovery of the hexane layer and 5) this method improves the repeatability of the results.

The linearity of all analytes was excellent and can be applied using only one standard level. We used the plasma pool as a secondary calibrator, since retinol, α-tocopherol and carotenoids have been reported to be stable in frozen serum from five months up to 4 years, depending on the temperature (Talwar et al. 1998; Comstock et al. 1995; Craft et al. 1988). In organic solvents, carotenoids become unstable (Su et al. 1999), thus increasing the variablility of the method. The inter-assay coefficient of variation was below 10% for all analytes using freezing, indicating excellent repeatability of the method. Absolute recoveries of carotenoids were at the same level as reported in previous studies (Lee et al. 2003; Talwar et al. 1998; Gueguen et al. 2002).

We connected two Synergy Hydro–RP80A columns in series in order to improve separation of lutein and zeaxanthin. Initially, we used one column, but zeaxanthin co-eluted with lutein, similar to other HPLC methods (Talwar et al. 1998; Gueguen et al. 2002; Nierenberg & Nann 1992). However, another type of column (e.g., C_{30}) would have further improved the selectivity (Rajendran et al. 2005). Lyan et al. also connected two columns in series, thereby increasing the selectivity and enabling successful separation of zeaxanthin from lutein, and canthaxanthin cis-isomers from trans-lycopene (Lyan et al. 2001).

We compared our HPLC method with the HPLC method at the Hospital Universitario Puerta de Hierro (Madrid, Spain) to confirm the reliability of the validated method. Correlation between the methods was adequate, except for zeaxanthin. The low correlation of zeaxanthin between methods may be due to partial coelution of zeaxanthin with lutein, even though calibration using peak height decreases any possible interference (work I, figure 2). Unfortunately, in our HPLC system, it was not possible to use the detector to verify the purity of the peaks with spectrophotometric wavelength scanning.

6.2 CONCENTRATIONS OF RETINOL, α-TOCOPHEROL AND CAROTENOIDS IN THE KIHD STUDY POPULATION AT 20-YEAR FOLLOW-UP

In this Eastern Finnish middle-aged KIHD study population, serum/plasma concentrations of carotenoids and α-tocopherol tended to increase, except for lycopene, over the 20-year follow-up period. Changes in plasma levels may be a consequence of dietary changes. From the late 1980s to the beginning of the 2000s, α-tocopherol values nearly doubled. For β-carotene and lycopene, we have the first values from the mid-1990s and the last values until the beginning of 2005, during

which plasma β-carotene had increased by 8%, while lycopene had decreased by 40%. It is possible that consumption of tomatoes or tomato products had decreased as the population grew older. Furthermore, the correlation between dietary intake and blood concentrations of lycopene has been reported to be poor (r = 0.19–0.47) (Scott et al. 1996; El-Sohemy et al. 2002). At the baseline and 11-year reexamination visit, dietary intake data was collected using a 4-day food diary and analysed for nutrients (Rissanen et al. 2003). We found that 4 days is too short a time to estimate the intake of carotenoids, since there was large day-to-day variation in the intake of the carotenoids, such as lycopene (Rissanen 2003). The plasma concentration of lycopene was lowest in 20 y (men) or 7 y (women) follow-up visit (the mean age for men and women was ~70 years). Seasonal variations in the concentrations of carotenoids were also observed. Lycopene concentrations tended to be highest between July and September, when tomato consumption is usually most abundant. Conversely, consumption of carrots seems to be highest in the late autumn and winter, reflecting the highest concentrations of α-carotene and β-carotene in the blood.

We cannot exclude the possibility that the decrease in lycopene levels from 11 y to 20 y (men) and from baseline to 7 y (women) is partly due to analytical changes. During the 20-year follow-up visit, the sample type was Li-heparin plasma, whereas the 4-year and 11-year visits collected samples from serum. It has been reported that no significant differences in the concentrations of carotenoids can be found between samples measured from serum or Li-heparin plasma, although these concentrations were slightly higher in serum (Olmedilla-Alonso et al. 2005). Another HPLC method was used in the 20-year follow-up for carotenoid analysis than had been used in previous follow-up visits. Peak-height was used in our previous and current method for quantification of carotenoids. We observed that total lycopene (cis+trans) was not measured in our previous follow-up visits. Therefore, difference in the levels of lycopene between methods was not significant under in vivo circumstances.

6.3 ROLE OF CAROTENOIDS IN LIPID OXIDATION

6.3.1 Effect of astaxanthin supplementation on lipid oxidation

In original work II, we found that astaxanthin supplementation of 8 mg/d may prevent lipid oxidation by decreasing the levels of plasma 12- and 15-hydroxy fatty acid, which are formed by oxidation of polyunsaturated linolenic and linolic acids. This decrease was almost significantly comparable to the placebo group. Fatty acids may be most sensitive to oxidation. C_{18} hydroxy fatty acids are good indicators during the first step in the oxidation of LDL lipids (Wang et al. 1992). In a study by Iwamoto and co-workers, 24 young healthy volunteers consumed 1.8-21.6 mg of astaxanthin/d for 2 weeks. The LDL lag time increased dose-dependently between days 0 and 14. The authors found no difference in the oxidation of LDL in the control group (Iwamoto et al. 2000). In a recent study, astaxanthin supplementation (2-8 mg for 4 week) decreased oxidative stress by

decreasing a DNA damage biomarker 8-hydroxy-2′-deoxyguanosine, but did not affect lipid oxidation (Park et al. 2010).

In a previous study, in which astaxanthin doses were greater than 8 mg/d, LDL lag time increased significantly (Iwamoto et al. 2000), suggesting that the daily dose of astaxanthin must be higher than 8 mg to achieve any impact on the lag time. However, astaxanthin supplementation did not have any effect on another in vivo lipid oxidation marker (free F_2 isoprostane concentration). Astaxanthin had no more effect on ex vivo lipid oxidation markers than did the placebo. The reasons why astaxanthin has no effect on other markers of lipid oxidation may include the young age of the subjects, who were healthy, non-smokers and had not been exposed to increased oxidative stress, or that the 8-mg supplement simply can not decrease lipid oxidation as measured by these markers.

Astaxanthin supplements were effectively absorbed from capsules into the systemic blood circulation and were found to be a safe dietary supplement, as it did not cause any gastrointestinal tract distress or other side effects. In previous supplementation studies, no side-effects, apart from headache, were observed, although astaxanthin doses were much higher than in our work (Osterlie et al. 2000; Coral-Hinostroza et al. 2004; Mercke Odeberg et al. 2003). Spiller et al. extensively studied the safety of astaxantin using a smaller daily dose (6 mg) than in our work and found no significant side-effects (Spiller & Dewell 2003).

6.3.2 Carotenoids and oxidative modification of LDL

Plasma lycopene, lutein and β-carotene were the most powerful antioxidants for explaining the content of in vivo oxidatively modified LDL in blood circulation (original work III).

Lycopene was the strongest determinant of serum LDL conjugated dienes in women, even though plasma lycopene concentration or its standard deviations did not differ between sexes. Moreover, lutein was an important antioxidant in women that decreased the concentrations of LDL conjugated dienes. In particular, β-carotene was the most efficient antioxidant in men. The low plasma concentration of β-carotene and other antioxidative vitamins in men may enhance men's endogenous antioxidative capacity, resulting in a lower oxidative status in vivo and lower conjugated diene content in LDL.

A natural carotenoid mixture increased the lag phase in Cu^{2+} induced oxidation of LDL. The beneficial effect may probably be a consequence of interaction between carotenoids (Kioskias & Gordon 2003). Lin and colleagues (Lin et al. 1998) have reported a decreased oxidation of LDL in healthy female non-smokers, who consumed a supplement rich in β-carotene and minor amounts of α-carotene, lycopene, zeaxanthin and lutein. This finding is in agreement with the notion that although a single antioxidant may have little influence on health, a combination of different antioxidants, as found in fruits and vegetables, may improve the effect. The combination of phenolic compounds and carotenoids has been shown to have synergistic effects by preventing human LDL oxidation more effectively than

carotenoids alone (Milde et al. 2007). Thus, co-operation between antioxidants may have affected the results of our study (III).

We were interested in the suitability of measuring LDL conjugated dienes as a marker of in vivo lipid oxidation. We wanted to examine what aspect could affect their formation. It was shown that all powerful antioxidants, such as α-tocopherol, observed in in vitro methods were not significant under in vivo circumstances.

6.4 ROLE OF LYCOPENE IN CANCER

In original work IV, we found that serum lycopene decreased the risk of cancer. The association between lycopene and prostate cancer risk has received the most attention (Rao & Rao 2007). Prostate cancer is the most commonly diagnosed malignancy in males (Cheung et al. 2008) and almost 40% of the cancers were prostate cancers in our cohort. The analysis was stratified to prostate cancer and other cancers to study whether the inverse relationship with serum lycopene would be similar for both groups. The association was observed for other cancers, but not for prostate cancer.

The mean blood concentration of lycopene has widely varied between different populations in Europe and USA (Table 2). The low blood level of lycopene is a consequence of a low dietary intake of tomatoes or tomato products or due to malabsorption or maldigestion (Drai et al. 2009), or possibly a result of increased oxidative stress in the body. Since our study population had very low concentrations of lycopene, but the concentration range was quite wide (0-1.02 μmol/l), statistical analysis showed that the protective effect against cancer was stronger than the results of site-specific cancers from other countries.

This is the first study to show a significant association between decreased serum lycopene levels and the risk of any cancer. Our results show that in Eastern Finnish men, a serum lycopene concentration >0.19 μmol/l may be a threshold value for decreasing the risk of cancer.

Two previous studies (Table 7) have reported a significant association between a low plasma concentration of lycopene and increased prostate cancer risk. These studies found that the protective plasma concentration of lycopene was >0.28 μmol/l (Lu et al. 2001) and >0.96 μmol/l (Zhang et al. 2007). Interestingly, the cutoff of the lowest plasma lycopene quartile (<0.18 μmol/l) (Lu et al. 2001) is nearly the same as the highest tertile (>0.19 μmol/l) in our Eastern Finnish population of men. Our results and those from previous studies suggest that a plasma lycopene concentration higher than about 0.2 μmol/l might present a threshold value for decreasing the risk of cancers, though this requires further research. In addition, the protective concentration of lycopene may depend on the type of the cancer.

Serum lycopene concentrations may provide an indicator for other beneficial dietary or lifestyle factors. In addition to lycopene, tomatoes contain numerous other phytochemicals, including carotenoids and polyphenols. Many of these nutrients and phytochemicals possess antioxidant and anticancer properties (Stacewicz-Sapuntzakis & Bowen 2005), and an interaction with lycopene may

contribute to the inverse associations found in our study. However, it would appear that the protective effect of lycopene on cancers seen in this study is not an over-estimate due to confounding of retinol, α-tocopherol, α-carotene and β-carotene, as they were not associated with the risk of cancer and their concentrations did not differ between the study groups. In addition, the correlation between blood lycopene concentrations and total intake of total vegetables and fruits has been shown to be very low (Campbell et al. 1994). Thus, blood lycopene may not simply be a reflection of a healthy diet (Rissanen 2003).

6.5 Strengths and limitations of the studies

The strengths of original work I include accurate validation of the HPLC method, method comparison, measurement of carotenoids that have not been analysed before in our laboratory. However, one limitation of this study is the poor correlation of zeaxanthin between the compared HPLC methods. Zeaxanthin peak purities could not be checked in our apparatus, and the resolution of lutein and zeaxanthin could be improved.

The strengths of original work II are its randomized double-blind design, use of numerous in vivo and in vitro lipid oxidation markers and the profound testing of astaxanthin supplement safety. If a larger effect of astaxanthin on lipid oxidation were desired, the astaxanthin dose would need to be higher than 8 mg to achieve any impact on most lipid oxidation markers. However, the 8 mg of astaxanthin per day corresponds to over 1.5 kg of farmed rainbow trout. Unfortunately, serum LDL conjugated dienes were not measured in the astaxanthin supplementation study.

In work III, the main determinants of serum LDL conjugated dienes were assessed and the carotenoids proved to be the most powerful determinants. The large range of possible determinants, including biochemical, anthropometric, behavioural and health measurements, were available to achieve reliable models for statistical testing. The lack of nutrient intake data is a limitation of this study. However, at the baseline and at the later follow-up visit of the KIHD, dietary intake data were collected using a 4-day food diary and concentrations of nutrients. We found 4 days to be too short a period of time to estimate the intake of some carotenoids, since there was a large day-to-day variation in the intake of carotenoids, such as lycopene. The human body may increase the endogenous antioxidative status in response to increased oxidative stress due to a low intake of dietary antioxidants or other unbalanced condition. Unfortunately, we did not have the possibility to measure endogenous plasma urate or glutathione or any other endogenous antioxidative enzymes, such as glutathione peroxidase, glutathione transferase and paraoxonase, or to evaluate their effect on serum LDL conjugated dienes.

The strengths of study IV include its prospective population-based design, complete follow-up (no losses), and reliable assessments of incident cancers. The limitations of this study might be that we included the variables of smoking at the

baseline in the model, even though we cannot exclude any possible residual confounding factors due to smoking, such as those related to a change in smoking habits after the baseline. The small numbers of cancer cases reduced the possibility of studying site-specific cancer risk, and the sum category of all non-prostate cancers may include cancer types with varying associations with lycopene.

7 Conclusions

The aim of the present study was to develop a rapid and simple method for analyzing carotenoids and evaluating their role in lipid oxidation and cancer. It was shown that plasma astaxanthin, lycopene, lutein and β-carotene may decrease oxidative modification of LDL (serum LDL conjugated dienes, C_{12} and C_{15} hydroxy fatty acids). Furthermore, serum lycopene may decrease the risk of cancer.

The main conclusions are:

1. The validated HPLC method for analysis of retinol, α-tocopherol and carotenoids (lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene) is simple, quick and repeatable for routine measurement of retinol, α-tocopherol and carotenoids.
2. Concentrations of α-tocopherol and carotenoids, except for lycopene, tended to increase in men and women, with increasing age, which may be explained by the increased consumption of fruits and vegetables that has occurred between the late 1980s and the beginning of the 2000s. However, it may be that elderly people consume less tomatoes and tomato products than do younger people, as indicated by the lower lycopene concentration found during the follow-up years in both sexes.
3. Astaxanthin supplemented as capsules was efficiently absorbed from the intestine into blood circulation, and was well tolerated. Nearly significant decrease was observed in the in vivo oxidation of fatty acids in healthy men.
4. Lycopene, lutein and β-carotene were the most powerful determinants for explaining the content of in vivo oxidatively modified LDL in serum.
5. In the population of Eastern Finnish men, high concentrations of serum lycopene were associated with a decreased risk of cancer. However, lycopene was not associated with prostate cancer in this population.

8 *References*

Agarwal S & Rao AV. Tomato lycopene and low density lipoprotein oxidation: a human dietary intervention study. Lipids 1998;33:981-984.

Ahuja KD, Ashton EL & Ball MJ. Effects of two lipid-lowering, carotenoid-controlled diets on the oxidative modification of low-density lipoproteins in free-living humans. Clin Sci 2003;105:355-361.

Albanes D, Heinonen OP, Huttunen JK, Taylor PR, Virtamo J, Edwards BK, Haapakoski J, Rautalahti M, Hartman AM & Palmgren J. Effects of alpha-tocopherol and beta-carotene supplements on cancer incidence in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study. Am J Clin Nutr 1995;62:1427S-1430S.

Albanes D, Heinonen OP, Taylor PR, Virtamo J, Edwards BK, Rautalahti M, Hartman AM, Palmgren J, Freedman LS, Haapakoski J, Barrett MJ, Pietinen P, Malila N, Tala E, Liippo K, Salomaa ER, Tangrea JA, Teppo L, Askin FB, Taskinen E, Erozan Y, Greenwald P & Huttunen JK. Alpha-Tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. J Natl Cancer Inst 1996;88:1560-1570.

Al-Delaimy WK, van Kappel AL, Ferrari P, Slimani N, Steghens JP, Bingham S, Johansson I, Wallstrom P, Overvad K, Tjonneland A, Key TJ, Welch AA, Bueno-de-Mesquita HB, Peeters PH, Boeing H, Linseisen J, Clavel-Chapelon F, Guibout C, Navarro C, Quiros JR, Palli D, Celentano E, Trichopoulou A, Benetou V, Kaaks R & Riboli E. Plasma levels of six carotenoids in nine European countries: report from the European Prospective Investigation into Cancer and Nutrition (EPIC). Public Health Nutr 2004;7:713-722.

Aman R, Schieber A & Carle R. Effects of heating and illumination on trans-cis isomerization and degradation of beta-carotene and lutein in isolated spinach chloroplasts. J Agric Food Chem 2005;53:9512-9518.

Ames BN, Gold LS & Willett WC. The causes and prevention of cancer. Proc Natl Acad Sci U S A 1995;92:5258-5265.

Andreoli R, Manini P, Poli D, Bergamaschi E, Mutti A & Niessen WM. Development of a simplified method for the simultaneous determination of retinol, alpha-tocopherol, and beta-carotene in serum by liquid chromatography-tandem

mass spectrometry with atmospheric pressure chemical ionization. Anal Bioanal Chem 2004;378:987-994.

Armstrong NC, Paganga G, Brunner E, Miller NJ, Nanchahal K, Shipley M, Rice-Evans CA, Marmot MG & Diplock AT. Reference values for alpha-tocopherol and beta-carotene in the Whitehall II Study. Free Radic Res 1997;27:207-219.

Astorg P. Food carotenoids and cancer prevention: an overview of current research. Trends Food Sci Technol 1997;8:406-413.

Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL & La Du BN. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A Possible peroxidative role for paraoxonase. J Clin Invest 1998;101:1581-1590.

Bandera EV, Freudenheim JL, Marshall JR, Zielezny M, Priore RL, Brasure J, Baptiste M & Graham S. Diet and alcohol consumption and lung cancer risk in the New York State Cohort (United States). Cancer Causes Control 1997;8:828-840.

Beckman JS & Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996;271:C1424-37.

Bertram JS. Carotenoids and gene regulation. Nutr Rev 1999;57:182-191.

Berzelius JJ. Concerning the yellow pigment in plant leaves. Annalen 1837;21:257-262.

Bhuvaneswari V & Nagini S. Lycopene: a review of its potential as an anticancer agent. Curr Med Chem Anticancer Agents 2005;5:627-635.

Bicanic D, Swarts J, Luterotti S, Pietraperzia G, Doka O & de Rooij H. Direct quantification of lycopene in products derived from thermally processed tomatoes: optothermal window as a selective, sensitive, and accurate analytical method without the need for preparatory steps. Anal Chem 2004;76:5203-5207.

Bohm F, Edge R, Burke M & Truscott TG. Dietary uptake of lycopene protects human cells from singlet oxygen and nitrogen dioxide - ROS components from cigarette smoke. J Photochem Photobiol B. 2001;64:176-178.

Bohm V & Bitsch R. Intestinal absorption of lycopene from different matrices and interactions to other carotenoids, the lipid status, and the antioxidant capacity of human plasma. Eur J Nutr 1999;38:118-125.

Bosetti C, Talamini R, Montella M, Negri E, Conti E, Franceschi S & La Vecchia C. Retinol, carotenoids and the risk of prostate cancer: a case-control study from Italy. Int J Cancer. 2004;112:689-692.

Bowen HT & Omaye ST. Oxidative changes associated with beta-carotene and alpha-tocopherol enrichment of human low-density lipoproteins. J Am Coll Nutr 1998;17:171-179.

Britton G. Structure and properties of carotenoids in relation to function. The FASEB J 1995;9:1551-1558.

Briviba K, Schnabele K, Rechkemmer G & Bub A. Supplementation of a diet low in carotenoids with tomato or carrot juice does not affect lipid peroxidation in plasma and feces of healthy men. J Nutr 2004;134:1081-1083.

Brown Thomas J, Duewer DL, Kline MC & Sharpless KE. The stability of retinol, alpha-tocopherol, trans-lycopene, and trans-beta-carotene in liquid-frozen and lyophilized serum. Clin Chim Acta 1998;276:75-87.

Buico A, Cassino C, Ravera M, Betta PG & Osella D. Oxidative stress and total antioxidant capacity in human plasma. Redox Rep 2009;14:125-131.

Buijsse B, Feskens EJ, Kwape L, Kok FJ & Kromhout D. Both alpha- and beta-carotene, but not tocopherols and vitamin C, are inversely related to 15-year cardiovascular mortality in Dutch elderly men. J Nutr 2008;138:344-350.

Burton GW & Ingold KU. beta-Carotene: an unusual type of lipid antioxidant. Science 1984;224:569-573.

Campbell DR, Gross MD, Martini MC, Grandits GA, Slavin JL & Potter JD. Plasma carotenoids as biomarkers of vegetable and fruit intake. Cancer Epidemiol Biomarkers Prev 1994;3:493-500.

Cantrell A, McGarvey DJ, Truscott TG, Rancan F & Bohm F. Singlet oxygen quenching by dietary carotenoids in a model membrane environment. Arch Biochem Biophys 2003;412:47-54.

Carpenter KL, van der Veen C, Hird R, Dennis IF, Ding T & Mitchinson MJ. The carotenoids beta-carotene, canthaxanthin and zeaxanthin inhibit macrophage-mediated LDL oxidation. FEBS lett 1997;401:262-266.

Carroll YL, Corridan BM & Morrissey PA. Lipoprotein carotenoid profiles and the susceptibility of low density lipoprotein to oxidative modification in healthy elderly volunteers. Eur J Clin Nutr 2000;54:500-507.

Chang S, Erdman JW,Jr, Clinton SK, Vadiveloo M, Strom SS, Yamamura Y, Duphorne CM, Spitz MR, Amos CI, Contois JH, Gu X, Babaian RJ, Scardino PT & Hursting SD. Relationship between plasma carotenoids and prostate cancer. Nutr cancer 2005;53:127-134.

Chatzimichalakis PF, Samanidou VF & Papadoyannis IN. Development of a validated liquid chromatography method for the simultaneous determination of eight fat-soluble vitamins in biological fluids after solid-phase extraction. J Chromatogr B Analyt Technol Biomed Life Sci 2004;805:289-296.

Chen L, Stacewicz-Sapuntzakis M, Duncan C, Sharifi R, Ghosh L, van Breemen R, Ashton D & Bowen PE. Oxidative DNA damage in prostate cancer patients consuming tomato sauce-based entrees as a whole-food intervention. J Natl Cancer Inst 2001;93:1872-1879.

Cheung E, Wadhera P, Dorff T & Pinski J. Diet and prostate cancer risk reduction. Expert Rev Anticancer Ther 2008;8:43-50.

Chopra M, O'Neill ME, Keogh N, Wortley G, Southon S & Thurnham DI. Influence of increased fruit and vegetable intake on plasma and lipoprotein carotenoids and LDL oxidation in smokers and nonsmokers. Clin Chem 2000;46:1818-1829.

Clark S, Youngman LD, Chukwurah B, Palmer A, Parish S, Peto R & Collins R. Effect of temperature and light on the stability of fat-soluble vitamins in whole blood over several days: implications for epidemiological studies. Int J Epidemiol 2004;33:518-525.

Colditz GA, Branch LG, Lipnick RJ, Willett WC, Rosner B, Posner BM & Hennekens CH. Increased green and yellow vegetable intake and lowered cancer deaths in an elderly population. Am J Clin Nutr 1985;41:32-36.

Comstock GW, Alberg AJ & Helzlsouer KJ. Reported effects of long-term freezer storage on concentrations of retinol, beta-carotene, and alpha-tocopherol in serum or plasma summarized. Clin Chem 1993;39:1075-1078.

Comstock GW, Norkus EP, Hoffman SC, Xu MW & Helzlsouer KJ. Stability of ascorbic acid, carotenoids, retinol, and tocopherols in plasma stored at -70 degrees C for 4 years. Cancer Epidemiol Biomarkers Prev 1995;4:505-507.

Conn PF, Lambert C, Land EJ, Schalch W & Truscott TG. Carotene-oxygen radical interactions. Free Radic Res Commun 1992;16:401-408.

Coral-Hinostroza GN, Ytrestoyl T, Ruyter B & Bjerkeng B. Plasma appearance of unesterified astaxanthin geometrical E/Z and optical R/S isomers in men given

single doses of a mixture of optical 3 and 3'R/S isomers of astaxanthin fatty acyl diesters. Comp Biochem Physiol C Toxicol Pharmacol 2004;139:99-110.

Correa Lima MP & Gomes-da-Silva MH. Colorectal cancer: lifestyle and dietary factors. Nutr Hosp 2005;20:235-241.

Craft NE, Brown ED & Smith JC,Jr. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem 1988;34:44-48.

Darley-Usmar V & Halliwell B. Blood radicals: reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. Pharm Res 1996;13:649-662.

de Groot H. Reactive oxygen species in tissue injury. Hepatogastroenterology 1994;41:328-332.

De Leenher AE, Lambert WE & Van Bocxlaer JF, etc. Modern Chromatographic Analysis of Vitamins, 2000.

Di Mascio P, Devasagayam TP, Kaiser S & Sies H. Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers. Biochem Soc Trans 1990;18:1054-1056.

Di Mascio P, Kaiser S & Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 1989;274:532-538.

Drai J, Borel P, Faure H, Galabert C, Le Moel G, Laromiguiere M & Fayol V. Fasting plasma carotenoids concentrations in Crohn's and pancreatic cancer patients compared to control subjects. Int J Vitam Nutr Res 2009;79:87-94.

Druesne-Pecollo N, Latino-Martel P, Norat T, Barrandon E, Bertrais S, Galan P & Hercberg S. Beta-carotene supplementation and cancer risk: a systematic review and metaanalysis of randomized controlled trials. Int J cancer 2010;127:172-184.

Dueker SR, Lunetta JM, Jones AD & Clifford AJ. Solid-phase extraction protocol for isolating retinol-d4 and retinol from plasma for parallel processing for epidemiological studies. Clin Chem 1993;39:2318-2322.

Dugas TR, Morel DW & Harrison EH. Impact of LDL carotenoid and alpha-tocopherol content on LDL oxidation by endothelial cells in culture. J Lipid Res 1998;39:999-1007.

Dwyer JH, Paul-Labrador MJ, Fan J, Shircore AM, Merz CN & Dwyer KM. Progression of carotid intima-media thickness and plasma antioxidants: the Los Angeles Atherosclerosis Study. Arterioscler Thromb Vasc Biol 2004;24:313-319.

Ehara S, Ueda M, Naruko T, Haze K, Itoh A, Otsuka M, Komatsu R, Matsuo T, Itabe H, Takano T, Tsukamoto Y, Yoshiyama M, Takeuchi K, Yoshikawa J & Becker AE. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 2001;103:1955-1960.

Elia M & Stratton RJ. Geographical inequalities in nutrient status and risk of malnutrition among English people aged 65 y and older. Nutrition 2005;21:1100-1106.

El-Sohemy A, Baylin A, Kabagambe E, Ascherio A, Spiegelman D & Campos H. Individual carotenoid concentrations in adipose tissue and plasma as biomarkers of dietary intake. Am J Clin Nutr 2002;76:172-179.

Erdman JW,Jr, Bierer TL & Gugger ET. Absorption and transport of carotenoids. Ann N Y Acad Sci 1993a;691:76-85.

Erdman JW,Jr, Bierer TL & Gugger ET. Absorption and transport of carotenoids. Ann N Y Acad Sci 1993b;691:76-85.

Etminan M, Takkouche B & Caamano-Isorna F. The role of tomato products and lycopene in the prevention of prostate cancer: a meta-analysis of observational studies. Cancer Epidemiol Biomarkers Prev 2004;13:340-345.

Finckh B, Kontush A, Commentz J, Hubner C, Burdelski M & Kohlschutter A. High-performance liquid chromatography-coulometric electrochemical detection of ubiquinol 10, ubiquinone 10, carotenoids, and tocopherols in neonatal plasma. Methods Enzymol1999;299:341-348.

Freese R, Alfthan G, Jauhiainen M, Basu S, Erlund I, Salminen I, Aro A & Mutanen M. High intakes of vegetables, berries, and apples combined with a high intake of linoleic or oleic acid only slightly affect markers of lipid peroxidation and lipoprotein metabolism in healthy subjects. Am J Clin Nutr 2002;76:950-960.

Galan P, Briancon S, Favier A, Bertrais S, Preziosi P, Faure H, Arnaud J, Arnault N, Czernichow S, Mennen L & Hercberg S. Antioxidant status and risk of cancer in the SU.VI.MAX study: is the effect of supplementation dependent on baseline levels? Br J Nutr 2005;94:125-132.

Gallicchio L, Boyd K, Matanoski G, Tao XG, Chen L, Lam TK, Shiels M, Hammond E, Robinson KA, Caulfield LE, Herman JG, Guallar E & Alberg AJ. Carotenoids and the risk of developing lung cancer: a systematic review. Am J Clin Nutr 2008;88:372-383.

Gann PH, Ma J, Giovannucci E, Willett W, Sacks FM, Hennekens CH & Stampfer MJ. Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis. Cancer Res 1999;59:1225-1230.

Garcia-Closas R, Berenguer A, Jose Tormo M, Jose Sanchez M, Quiros JR, Navarro C, Arnaud R, Dorronsoro M, Dolores Chirlaque M, Barricarte A, Ardanaz E, Amiano P, Martinez C, Agudo A & Gonzalez CA. Dietary sources of vitamin C, vitamin E and specific carotenoids in Spain. Br J Nutr 2004;91:1005-1011.

Gaziano JM, Hatta A, Flynn M, Johnson EJ, Krinsky NI, Ridker PM, Hennekens CH & Frei B. Supplementation with beta-carotene in vivo and in vitro does not inhibit low density lipoprotein oxidation. Atherosclerosis 1995;112:187-195.

Giovannucci E, Ascherio A, Rimm EB, Stampfer MJ, Colditz GA & Willett WC. Intake of carotenoids and retinol in relation to risk of prostate cancer. J Natl Cancer Inst 1995;87:1767-1776.

Goralczyk K. Beta-carotene and lung cancer in smokers: review of hypothesis and status of research. Nutr Cancer 2009;61:767-774.

Goto S, Kogure K, Abe K, Kimata Y, Kitahama K, Yamashita E & Terada H. Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. Biochim Biophys Acta 2001;1512:251-258.

Granado F, Blazquez S & Olmedilla B. Changes in carotenoid intake from fruit and vegetables in the Spanish population over the period 1964-2004. Public health Nutr 2007;10:1018-1023.

Granado F, Olmedilla B, Blanco I & Rojas-Hidalgo E. Major fruit and vegetable contributors to the main serum carotenoids in the Spanish diet. Eur J Clin Nutr 1996;50:246-250.

Granado-Lorencio F, Olmedilla-Alonso B, Herrero-Barbudo C, Perez-Sacristan B, Blanco-Navarro I & Blazquez-Garcia S. Comparative in vitro bioaccessibility of carotenoids from relevant contributors to carotenoid intake. J Agric Food Chem 2007;55:6387-6394.

Gueguen S, Herbeth B, Siest G & Leroy P. An isocratic liquid chromatographic method with diode-array detection for the simultaneous determination of alpha-tocopherol, retinol, and five carotenoids in human serum. J Chromatogr Sci 2002;40:69-76.

Guerin M, Huntley ME & Olaizola M. Haematococcus astaxanthin: applications for human health and nutrition. Trends biotechnol 2003;21:210-216.

Han RM, Chen CH, Tian YX, Zhang JP & Skibsted LH. Fast regeneration of carotenoids from radical cations by isoflavonoid dianions: importance of the carotenoid keto group for electron transfer. J Phys Chem A 2010;114:126-132.

Handelman GJ, Dratz EA, Reay CC & van Kuijk JG. Carotenoids in the human macula and whole retina. Invest Ophthalmol Vis Sci 1988;29:850-855.

Hermans N, Cos P, Berghe DV, Vlietinck AJ & de Bruyne T. Method development and validation for monitoring in vivo oxidative stress: evaluation of lipid peroxidation and fat-soluble vitamin status by HPLC in rat plasma. Cancer Epidemiol Biomarkers Prev 2005;822:33-39.

Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT & Ala-Korpela M. Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim Biophys Acta 2000;1488:189-210.

Hininger IA, Meyer-Wenger A, Moser U, Wright A, Southon S, Thurnham D, Chopra M, Van Den Berg H, Olmedilla B, Favier AE & Roussel AM. No significant effects of lutein, lycopene or beta-carotene supplementation on biological markers of oxidative stress and LDL oxidizability in healthy adult subjects. J Am Coll Nutr 2001;20:232-238.

Hornero-Mendez D & Minguez-Mosquera MI. Rapid spectrophotometric determination of red and yellow isochromic carotenoid fractions in paprika and red pepper oleoresins. J Agric Food Chem 2001;49:3584-3588.

Huang HY, Alberg AJ, Norkus EP, Hoffman SC, Comstock GW & Helzlsouer KJ. Prospective study of antioxidant micronutrients in the blood and the risk of developing prostate cancer. Am J Epidemiol 2003;157:335-344.

Huck CW, Popp M, Scherz H & Bonn GK. Development and evaluation of a new method for the determination of the carotenoid content in selected vegetables by HPLC and HPLC-MS-MS. J Chromatogr Sci 2000;38:441-449.

Hung RJ, Zhang ZF, Rao JY, Pantuck A, Reuter VE, Heber D & Lu QY. Protective effects of plasma carotenoids on the risk of bladder cancer. J Urol 2006;176:1192-1197.

Hwang ES & Bowen PE. Effects of lycopene and tomato paste extracts on DNA and lipid oxidation in LNCaP human prostate cancer cells. Biofactors 2005;23:97-105.

Itabe H. Oxidative modification of LDL: its pathological role in atherosclerosis. Clin Rev Allergy Immunol 2009;37:4-11.

Itabe H. Oxidized low-density lipoproteins: what is understood and what remains to be clarified. Biol Pharm Bull 2003;26:1-9.

Itabe H. Oxidized phospholipids as a new landmark in atherosclerosis. Prog Lipid Res 1998;37:181-207.

Iwamoto T, Hosoda K, Hirano R, Kurata H, Matsumoto A, Miki W, Kamiyama M, Itakura H, Yamamoto S & Kondo K. Inhibition of low-density lipoprotein oxidation by astaxanthin. J Atheroscler Thromb 2000;7:216-222.

Jackson H, Braun CL & Ernst H. The chemistry of novel xanthophyll carotenoids. Am J Cardiol 2008;101:50D-57D.

Jenab M, Riboli E, Ferrari P, Friesen M, Sabate J, Norat T, Slimani N, Tjonneland A, Olsen A, Overvad K, Boutron-Ruault MC, Clavel-Chapelon F, Boeing H, Schulz M, Linseisen J, Nagel G, Trichopoulou A, Naska A, Oikonomou E, Berrino F, Panico S, Palli D, Sacerdote C, Tumino R, Peeters PH, Numans ME, Bueno-de-Mesquita HB, Buchner FL, Lund E, Pera G, Chirlaque MD, Sanchez MJ, Arriola L, Barricarte A, Quiros JR, Johansson I, Johansson A, Berglund G, Bingham S, Khaw KT, Allen N, Key T, Carneiro F, Save V, Del Giudice G, Plebani M, Kaaks R & Gonzalez CA. Plasma and dietary carotenoid, retinol and tocopherol levels and the risk of gastric adenocarcinomas in the European prospective investigation into cancer and nutrition. Br J Cancer 2006;95:406-415.

Jewell C & O'Brien NM. Effect of dietary supplementation with carotenoids on xenobiotic metabolizing enzymes in the liver, lung, kidney and small intestine of the rat. Br J Nutr 1999;81:235-242.

Jialal I, Norkus EP, Cristol L & Grundy SM. beta-Carotene inhibits the oxidative modification of low-density lipoprotein. Biochim Biophys Acta 1991;1086:134-138.

Jian L, Du CJ, Lee AH & Binns CW. Do dietary lycopene and other carotenoids protect against prostate cancer? Int J Cancer 2005;113:1010-1014.

Jiang J, Suzuki S, Xiang J, Kuriki K, Hosono A, Arakawa K, Wang J, Nagaya T, Kojima M, Katsuda N & Tokudome S. Plasma carotenoid, alpha-tocopherol and retinol concentrations and risk of colorectal adenomas: A case-control study in Japan. Cancer lett 2005;226:133-141.

Jordan P, Brubacher D, Moser U, Stahelin HB & Gey KF. Vitamin E and vitamin A concentrations in plasma adjusted for cholesterol and triglycerides by multiple regression. Clin Chem 1995;41:924-927.

Kaikkonen J, Nyyssönen K, Porkkala-Sarataho E, Poulsen HE, Metsä-Ketelä T, Hayn M, Salonen R & Salonen JT. Effect of oral coenzyme Q10 supplementation on the oxidation resistance of human VLDL+LDL fraction: absorption and antioxidative properties of oil and granule-based preparations. Free Radic Biol Med 1997;22:1195-1202.

Karas M, Amir H, Fishman D, Danilenko M, Segal S, Nahum A, Koifmann A, Giat Y, Levy J & Sharoni Y. Lycopene interferes with cell cycle progression and insulin-like growth factor I signaling in mammary cancer cells. Nutr cancer 2000;36:101-111.

Kavanaugh CJ, Trumbo PR & Ellwood KC. The U.S. Food and Drug Administration's evidence-based review for qualified health claims: tomatoes, lycopene, and cancer. J Nat Cancer Inst 2007;99:1074-1085.

Kelm MA, Flanagan VP, Pawlosky RJ, Novotny JA, Clevidence BA & Britz SJ. Quantitative determination of 13C-labeled and endogenous beta-carotene, lutein, and vitamin A in human plasma. Lipids 2001;36:1277-1282.

Key TJ, Appleby PN, Allen NE, Travis RC, Roddam AW, Jenab M, Egevad L, Tjonneland A, Johnsen NF, Overvad K, Linseisen J, Rohrmann S, Boeing H, Pischon T, Psaltopoulou T, Trichopoulou A, Trichopoulos D, Palli D, Vineis P, Tumino R, Berrino F, Kiemeney L, Bueno-de-Mesquita HB, Quiros JR, Gonzalez CA, Martinez C, Larranaga N, Chirlaque MD, Ardanaz E, Stattin P, Hallmans G, Khaw KT, Bingham S, Slimani N, Ferrari P, Rinaldi S & Riboli E. Plasma carotenoids, retinol, and tocopherols and the risk of prostate cancer in the European Prospective Investigation into Cancer and Nutrition study. Am J Clin Nutr 2007;86:672-681.

Khachik F, Spangler CJ, Smith JC,Jr, Canfield LM, Steck A & Pfander H. Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. Anal Chem 1997;69:1873-1881.

Kioskias S & Gordon MH. Dietary supplementation with a natural carotenoid mixture decreases oxidative stress. Eur J Clin Nutr 2003;57:1135-1140.

Kirsh VA, Mayne ST, Peters U, Chatterjee N, Leitzmann MF, Dixon LB, Urban DA, Crawford ED & Hayes RB. A prospective study of lycopene and tomato product intake and risk of prostate cancer. Cancer Epidemiol Biomarkers Prev 2006;15:92-98.

Kleemola P, Freese R, Jauhiainen M, Pahlman R, Alfthan G & Mutanen M. Dietary determinants of serum paraoxonase activity in healthy humans. Atherosclerosis 2002;160:425-432.

Kleinsmith LJ. Principles of cancer biology. San Francisco: Pearson Benjamin Cummings. 2006.

Knekt P, Ritz J, Pereira MA, O'Reilly EJ, Augustsson K, Fraser GE, Goldbourt U, Heitmann BL, Hallmans G, Liu S, Pietinen P, Spiegelman D, Stevens J, Virtamo J, Willett WC, Rimm EB & Ascherio A. Antioxidant vitamins and coronary heart disease risk: a pooled analysis of 9 cohorts. Am J Clin Nutr 2004;80:1508-1520.

Kohlmeier L & Hastings SB. Epidemiologic evidence of a role of carotenoids in cardiovascular disease prevention. Am J Clin Nutr 1995;62:1370S-1376S.

Krinsky NI & Johnson EJ. Carotenoid actions and their relation to health and disease. Mol Aspects Med 2005;26:459-516.

Krinsky NI & Yeum KJ. Carotenoid-radical interactions. Biochem Biophys Res Comm 2003;305:754-760.

Kucuk O, Sarkar FH, Sakr W, Djuric Z, Pollak MN, Khachik F, Li YW, Banerjee M, Grignon D, Bertram JS, Crissman JD, Pontes EJ & Wood DP,Jr. Phase II randomized clinical trial of lycopene supplementation before radical prostatectomy. Mol Aspects Med 2001;10:861-868.

Kushi LH, Kwan ML, Lee MM & Ambrosone CB. Lifestyle factors and survival in women with breast cancer. J Nutr 2007;137:236S-242S.

Laukkanen JA, Laaksonen DE, Niskanen L, Pukkala E, Hakkarainen A & Salonen JT. Metabolic syndrome and the risk of prostate cancer in Finnish men: a population-based study. Cancer Epidemiol Biomarkers Prev 2004;13:1646-1650.

Lauretani F, Semba RD, Dayhoff-Brannigan M, Corsi AM, Di Iorio A, Buiatti E, Bandinelli S, Guralnik JM & Ferrucci L. Low total plasma carotenoids are independent predictors of mortality among older persons: the InCHIANTI study. Eur J Nutr 2008;47:335-340.

Lee BL & Ong CN. Comprehensive high-performance liquid chromatographic method for the measurements of lipophilic antioxidants in human plasma. J Chromatogr A 2009;1216:3131-3137.

Lee BL, New AL & Ong CN. Simultaneous determination of tocotrienols, tocopherols, retinol, and major carotenoids in human plasma. Clin Chem 2003;49:2056-2066.

Lee JE, Männisto S, Spiegelman D, Hunter DJ, Bernstein L, van den Brandt PA, Buring JE, Cho E, English DR, Flood A, Freudenheim JL, Giles GG, Giovannucci E, Hakansson N, Horn-Ross PL, Jacobs EJ, Leitzmann MF, Marshall JR, McCullough ML, Miller AB, Rohan TE, Ross JA, Schatzkin A, Schouten LJ, Virtamo J, Wolk A, Zhang SM & Smith-Warner SA. Intakes of fruit, vegetables, and carotenoids and renal cell cancer risk: a pooled analysis of 13 prospective studies. Cancer Epidemiol Biomarkers Prev 2009a;18:1730-1739.

Lee JE, Männisto S, Spiegelman D, Hunter DJ, Bernstein L, van den Brandt PA, Buring JE, Cho E, English DR, Flood A, Freudenheim JL, Giles GG, Giovannucci E, Hakansson N, Horn-Ross PL, Jacobs EJ, Leitzmann MF, Marshall JR, McCullough ML, Miller AB, Rohan TE, Ross JA, Schatzkin A, Schouten LJ, Virtamo J, Wolk A, Zhang SM & Smith-Warner SA. Intakes of fruit, vegetables, and carotenoids and renal cell cancer risk: a pooled analysis of 13 prospective studies. Cancer Epidemiol Biomarkers Prev 2009b;18:1730-1739.

Li L, Chen CY, Aldini G, Johnson EJ, Rasmussen H, Yoshida Y, Niki E, Blumberg JB, Russell RM & Yeum KJ. Supplementation with lutein or lutein plus green tea extracts does not change oxidative stress in adequately nourished older adults. J Nutr Biochem 2010;21(6):544-9.

Liang R, Han RM, Fu LM, Ai XC, Zhang JP & Skibsted LH. Baicalin in radical scavenging and its synergistic effect with beta-carotene in antilipoxidation. J Agric Food Chem 2009;57:7118-7124.

Lin Y, Burri BJ, Neidlinger TR, Muller H- & Dueker, S.R. & Clifford, A.J. Estimating the concentration of β-carotene required for maximal protection of low-density lipoproteins in women. Am J Clin Nutr 1998;67:837-845.

Lindahl T. Instability and decay of the primary structure of DNA. Nature 1993;362:709-715.

Liu C, Wang XD, Mucci L, Gaziano JM & Zhang SM. Modulation of lung molecular biomarkers by beta-carotene in the Physicians' Health Study. Cancer 2009;115:1049-1058.

Livny O, Reifen R, Levy I, Madar Z, Faulks R, Southon S & Schwartz B. Beta-carotene bioavailability from differently processed carrot meals in human ileostomy volunteers. Eur J Nutr 2003;42:338-345.

Lorenzo Y, Azqueta A, Luna L, Bonilla F, Dominguez G & Collins AR. The carotenoid beta-cryptoxanthin stimulates the repair of DNA oxidation damage in addition to acting as an antioxidant in human cells. Carcinogenesis 2009;30:308-314.

Lornejad-Schafer MR, Lambert C, Breithaupt DE, Biesalski HK & Frank J. Solubility, uptake and biocompatibility of lutein and zeaxanthin delivered to cultured human retinal pigment epithelial cells in tween40 micelles. Eur J Nutr 2007;46:79-86.

Lowe GM, Vlismas K & Young AJ. Carotenoids as prooxidants? Mol Aspects Med 2003;24:363-369.

Lu QY, Hung JC, Heber D, Go VL, Reuter VE, Cordon-Cardo C, Scher HI, Marshall JR & Zhang ZF. Inverse associations between plasma lycopene and other carotenoids and prostate cancer. Cancer Epidemiol Biomarkers Prev 2001;10:749-756.

Lucarini M, Lanzi S, D'Evoli L, Aguzzi A & Lombardi-Boccia G. Intake of vitamin A and carotenoids from the Italian population--results of an Italian total diet study. Int J Vitam Nutr Res 2006;76:103-109.

Lyan B, Azais-Braesco V, Cardinault N, Tyssandier V, Borel P, Alexandre-Gouabau MC & Grolier P. Simple method for clinical determination of 13 carotenoids in human plasma using an isocratic high-performance liquid chromatographic method. Journal J Chromatogr B Analyt Technol Biomed Life Sci 2001;751:297-303.

Mackness MI, Harty D, Bhatnagar D, Winocour PH, Arrol S, Ishola M & Durrington PN. Serum paraoxonase activity in familial hypercholesterolaemia and insulin-dependent diabetes mellitus. Atherosclerosis 1991;86:193-199.

Maiani G, Caston MJ, Catasta G, Toti E, Cambrodon IG, Bysted A, Granado-Lorencio F, Olmedilla-Alonso B, Knuthsen P, Valoti M, Bohm V, Mayer-Miebach E, Behsnilian D & Schlemmer U. Carotenoids: actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. Mol Nutr Food Res 2009;53 Suppl 2:S194-218.

Mayne ST, Cartmel B, Lin H, Zheng T & Goodwin WJ,Jr. Low plasma lycopene concentration is associated with increased mortality in a cohort of patients with prior oral, pharynx or larynx cancers. J Am Coll Nutr 2004;23:34-42.

McCall MR & Frei B. Can antioxidant vitamins materially reduce oxidative damage in humans? Free Radic Biol Med 1999;26:1034-1053.

McGeachin RB & Bailey CA. Determination of carotenoid pigments, retinol, and alpha-tocopherol in feeds, tissues, and blood serum by normal phase high performance liquid chromatography. Poult Sci 1995;74:407-411.

Mercke Odeberg J, Lignell A, Pettersson A & Hoglund P. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. Eur J Pharm Sci 2003;19:299-304.

Milde J, Elstner EF & Grassmann J. Synergistic effects of phenolics and carotenoids on human low-density lipoprotein oxidation. Mol Nutr Food Res 2007;51:956-961.

Milde J, Elstner EF & Grassmann J. Synergistic inhibition of low-density lipoprotein oxidation by rutin, gamma-terpinene, and ascorbic acid. Int J Phytother Phytopharm 2004;11:105-113.

Mineraalilaboratorio MILA. Available: http://www.mineraalilaboratoriomila.fi/.

Montonen J, Knekt P, Jarvinen R & Reunanen A. Dietary antioxidant intake and risk of type 2 diabetes. Diabetes Care 2004;27:362-366.

Morrow JD, Roberts LJ 2nd. Mass spectrometric quantification of F2-isoprostanes in biological fluids and tissues as measure of oxidant stress.Methods Enzymol 1999;300:3-12.

Mortensen A & Skibsted LH. Relative stability of carotenoid radical cations and homologue tocopheroxyl radicals. A real time kinetic study of antioxidant hierarchy. FEBS lett 1997;417:261-266.

Mugge A. The role of reactive oxygen species in atherosclerosis. Z Kardiol 1998;87:851-864.

Männisto S, Yaun SS, Hunter DJ, Spiegelman D, Adami HO, Albanes D, van den Brandt PA, Buring JE, Cerhan JR, Colditz GA, Freudenheim JL, Fuchs CS, Giovannucci E, Goldbohm RA, Harnack L, Leitzmann M, McCullough ML, Miller AB, Rohan TE, Schatzkin A, Virtamo J, Willett WC, Wolk A, Zhang SM & Smith-Warner SA. Dietary carotenoids and risk of colorectal cancer in a pooled analysis of 11 cohort studies. Am J Epidemiol 2007;165:246-255.

Nagao A. Absorption and function of dietary carotenoids. Forum Nutr 2009;61:55-63.

Naguib YM. Antioxidant activities of astaxanthin and related carotenoids. J Agric Food Chem 2000;48:1150-1154.

Naruko T, Ueda M, Ehara S, Itoh A, Haze K, Shirai N, Ikura Y, Ohsawa M, Itabe H, Kobayashi Y, Yamagishi H, Yoshiyama M, Yoshikawa J & Becker AE. Persistent high levels of plasma oxidized low-density lipoprotein after acute myocardial infarction predict stent restenosis. Arterioscler Thromb Vasc Biol 2006;26:877-883.

Nierenberg DW & Nann SL. A method for determining concentrations of retinol, tocopherol, and five carotenoids in human plasma and tissue samples. Am J Clin Nutr 1992;56:417-426.

Nishino H, Murakoshi M, Tokuda H & Satomi Y. Cancer prevention by carotenoids. Arch Biochem Biophys 2009;483:165-168.

Nomura AM, Stemmermann GN, Lee J & Craft NE. Serum micronutrients and prostate cancer in Japanese Americans in Hawaii. Cancer Epidemiol Biomarkers Prev 1997;6:487-491.

Nyyssönen K, Porkkala-Sarataho E, Kaikkonen J & Salonen JT. Ascorbate and urate are the strongest determinants of plasma antioxidative capacity and serum lipid resistance to oxidation in Finnish men. Atherosclerosis 1997;130:223-233.

Obermuller-Jevic UC, Olano-Martin E, Corbacho AM, Eiserich JP, van der Vliet A, Valacchi G, Cross CE & Packer L. Lycopene inhibits the growth of normal human prostate epithelial cells in vitro. J Nut 2003;133:3356-3360.

Olmedilla B, Granado F, Gil-Martinez E, Blanco I & Rojas-Hidalgo E. Reference values for retinol, tocopherol, and main carotenoids in serum of control and insulin-dependent diabetic Spanish subjects. Clin Chem 1997;43:1066-1071.

Olmedilla B, Granado F, Southon S, Wright AJ, Blanco I, Gil-Martinez E, Berg H, Corridan B, Roussel AM, Chopra M & Thurnham DI. Serum concentrations of carotenoids and vitamins A, E, and C in control subjects from five European countries. Br J Nutr 2001;85:227-238.

Olmedilla-Alonso B, Granado-Lorencio F & Blanco-Navarro I. Carotenoids, retinol and tocopherols in blood: comparability between serum and plasma (Li-heparin) values. Clin Biochem 2005;38:444-449.

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S & Hammar S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N Engl J Med 1996;334:1150-1155.

O'Neill ME, Carroll Y, Corridan B, Olmedilla B, Granado F, Blanco I, Van den Berg H, Hininger I, Rousell AM, Chopra M, Southon S & Thurnham DI. A European carotenoid database to assess carotenoid intakes and its use in a five-country comparative study. Br J Nutr 2001;85:499-507.

Ortega H, Coperias JL, Castilla P, Gomez-Coronado D & Lasuncion MA. Liquid chromatographic method for the simultaneous determination of different lipid-soluble antioxidants in human plasma and low-density lipoproteins. J Chromatogr B Analyt Technol Biomed Life Sci 2004;803:249-255.

Osganian SK, Stampfer MJ, Rimm E, Spiegelman D, Manson JE & Willett WC. Dietary carotenoids and risk of coronary artery disease in women. Am J Clin Nutr 2003;77:1390-1399.

Osterlie M, Bjerkeng B & Liaaen-Jensen S. Plasma appearance and distribution of astaxanthin E/Z and R/S isomers in plasma lipoproteins of men after single dose administration of astaxanthin. J Nutr Biochem 2000;11:482-490.

Paiva SA & Russell RM. Beta-carotene and other carotenoids as antioxidants. J Am Coll Nutr 1999;18:426-433.

Palozza P & Krinsky NI. Antioxidant effects of carotenoids in vivo and in vitro: an overview. Methods Ezymol 1992;213:403-420.

Palozza P, Luberto C, Calviello G, Ricci P & Bartoli GM. Antioxidant and prooxidant role of beta-carotene in murine normal and tumor thymocytes: effects of oxygen partial pressure. Free Radic Biol Med 1997;22:1065-1073.

Park JS, Chyun JH, Kim YK, Line LL & Chew BP. Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. Nutr Metab 2010;7:18.

Parker RS, Swanson JE, You CS, Edwards AJ & Huang T. Bioavailability of carotenoids in human subjects. Proc Nutr Soc 1999;58:155-162.

Parker RS. Absorption, metabolism, and transport of carotenoids. The FASEB J 1996;10:542-551.

Parviainen MT, Nyyssönen K, Penttilä IM, Seppänen K, Rauramaa R, Salonen J & Gref GG. A method for routine assay of plasma ascorbic acid using high-performance liquid chromatography. J Liq Chromatogr 1986;9:2185-2197.

Paterson E, Gordon MH, Niwat C, George TW, Parr L, Waroonphan S & Lovegrove JA. Supplementation with fruit and vegetable soups and beverages

increases plasma carotenoid concentrations but does not alter markers of oxidative stress or cardiovascular risk factors. J Nutr 2006;136:2849-2855.

Peng YS, Peng YM, McGee DL & Alberts DS. Carotenoids, tocopherols, and retinoids in human buccal mucosal cells: intra- and interindividual variability and storage stability. Am J Clin Nutr 1994;59:636-643.

Persson C, Sasazuki S, Inoue M, Kurahashi N, Iwasaki M, Miura T, Ye W, Tsugane S & JPHC Study Group. Plasma levels of carotenoids, retinol and tocopherol and the risk of gastric cancer in Japan: a nested case-control study. Carcinogenesis 2008;29:1042-1048.

Peters U, Leitzmann MF, Chatterjee N, Wang Y, Albanes D, Gelmann EP, Friesen MD, Riboli E & Hayes RB. Serum lycopene, other carotenoids, and prostate cancer risk: a nested case-control study in the prostate, lung, colorectal, and ovarian cancer screening trial. Cancer Epidemiol Biomarkers Prev 2007;16:962-968.

Porkkala-Sarataho E, Nyyssönen K & Salonen JT. Increased oxidation resistance of atherogenic plasma lipoproteins at high vitamin E levels in non-vitamin E supplemented men. Atherosclerosis 1996;124:83-94.

Poulsen HE, Jensen BR, Weimann A, Jensen SA, Sorensen M & Loft S. Antioxidants, DNA damage and gene expression. Free Radic Res 2000;33 Suppl:S33-9.

Pryor WA & Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol 1995;268:L699-722.

Rajendran V, Pu YS & Chen BH. An improved HPLC method for determination of carotenoids in human serum. J Chromatogr B Analyt Technol Biomed Life Sci 2005;824:99-106.

Rao AV & Rao LG. Carotenoids and human health. Pharm Res 2007;55:207-216.

Rao AV, Ray MR & Rao LG. Lycopene. Adv Food Nutr Res 2006;51:99-164.

Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA & Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. N Engl J Med 1993;328:1450-1456.

Rissanen T. Association of lycopene and dietary intake of fruits, berries and vegetables with atherosclerosis and cardiovascular diseases: epidemiologic evidence. Kuopio: Kuopion yliopisto 2003.

Rissanen TH, Voutilainen S, Nyyssönen K, Lakka TA, Sivenius J, Salonen R, Kaplan GA & Salonen JT. Low serum lycopene concentration is associated with an excess incidence of acute coronary events and stroke: the Kuopio Ischaemic Heart Disease Risk Factor Study. Br J Nutr 2001;85:749-754.

Rissanen TH, Voutilainen S, Virtanen JK, Venho B, Vanharanta M, Mursu J & Salonen JT. Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. J Nutr 2003;133:199-204.

Salonen JT, Salonen R, Seppänen K, Rauramaa R & Tuomilehto J. HDL, HDL2, and HDL3 subfractions, and the risk of acute myocardial infarction. A prospective population study in Eastern Finnish men. Circulation 1991;84:129-139.

Sander LC, Sharpless KE, Craft NE & Wise SA. Development of engineered stationary phases for the separation of carotenoid isomers. Anal Chem 1994;66:1667-1674.

Satia JA, Littman A, Slatore CG, Galanko JA & White E. Long-term use of beta-carotene, retinol, lycopene, and lutein supplements and lung cancer risk: results from the VITamins And Lifestyle (VITAL) study. Am J Epidemiol 2009;169:815-828.

Schreier L, Pagliero F, Sanguinetti S, Wikinski R. influence of the medium on the assessment of LDL resistance to oxidation: lag time in phosphate buffered saline is longer than in sodium chloride solution. Atherosclerosis 1997;129:127-128.

Schuurman AG, Goldbohm RA, Brants HA & van den Brandt PA. A prospective cohort study on intake of retinol, vitamins C and E, and carotenoids and prostate cancer risk (Netherlands). Cancer Causes Control 2002;13:573-582.

Schwarz S, Obermuller-Jevic UC, Hellmis E, Koch W, Jacobi G & Biesalski HK. Lycopene inhibits disease progression in patients with benign prostate hyperplasia. J Nutr 2008;138:49-53.

Schwedhelm E, Maas R, Troost R & Boger RH. Clinical pharmacokinetics of antioxidants and their impact on systemic oxidative stress. Clin Pharmacokinet 2003;42:437-459.

Schweigert FJ, Steinhagen B, Raila J, Siemann A, Peet D & Buscher U. Concentrations of carotenoids, retinol and alpha-tocopherol in plasma and follicular fluid of women undergoing IVF. Hum Reprod 2003;18:1259-1264.

Scott KJ, Thurnham DI, Hart DJ, Bingham SA & Day K. The correlation between the intake of lutein, lycopene and beta-carotene from vegetables and fruits, and blood plasma concentrations in a group of women aged 50-65 years in the UK. Br J Nutr 1996;75:409-418.

Seren S, Lieberman R, Bayraktar UD, Heath E, Sahin K, Andic F & Kucuk O. Lycopene in cancer prevention and treatment. Am J Ther 2008;15:66-81.

Sesso HD, Buring JE, Norkus EP & Gaziano JM. Plasma lycopene, other carotenoids, and retinol and the risk of cardiovascular disease in men. Am J Clin Nutr 2005;81:990-997.

Sesso HD, Buring JE, Norkus EP & Gaziano JM. Plasma lycopene, other carotenoids, and retinol and the risk of cardiovascular disease in women. Am J Clin Nutr 2004;79:47-53.

Shibutani S, Takeshita M & Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature 1991;349:431-434.

Shils ME, Shike M, Ross AC, Caballero B and Cousins RJ. Modern Nutrition in Health and Disease, 2006.

Slattery ML, Benson J, Curtin K, Ma KN, Schaeffer D & Potter JD. Carotenoids and colon cancer. Am J Clin Nutr 2000;71:575-582.

Spiller GA & Dewell A. Safety of an astaxanthin-rich Haematococcus pluvialis algal extract: a randomized clinical trial. J Med food 2003;6:51-56.

Stacewicz-Sapuntzakis M & Bowen PE. Role of lycopene and tomato products in prostate health. Biochim Biophys Acta 2005;1740:202-205.

Stahl W & Sies H. Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans. J Nutr 1992;122:2161-2166.

Stahl W, Schwarz W, Sundquist AR & Sies H. Cis-Trans Isomers of Lycopene and Beta-Carotene in Human Serum and Tissues. Arch Biochem Biophys 1992;294:173-177.

Stanner SA, Hughes J, Kelly CN & Buttriss J. A review of the epidemiological evidence for the 'antioxidant hypothesis'. Public Health Nutr 2004;7:407-422.

Steinberg D. The LDL modification hypothesis of atherogenesis: an update. J Lipid Res 2009;50 Suppl:S376-81.

Su Q, Rowley KG & Balazs ND. Carotenoids: separation methods applicable to biological samples. J Chromatogr B Analyt Technol Biomed Life Sci 2002;781:393-418.

Su Q, Rowley KG & O'Dea K. Stability of individual carotenoids, retinol and tocopherols in human plasma during exposure to light and after extraction. J Chromatogr B Analyt Technol Biomed Life Sci 1999;729:191-198.

Tabacchi G, Wijnhoven TM, Branca F, Román-Viñas B, Ribas-Barba L, Ngo J, García-Alvarez A, Serra-Majem L. How is the adequacy of micronutrient intake assessed across Europe? A systematic literature review. Br J Nutr. 2009;101:S29-36.

Talwar D, Ha TK, Cooney J, Brownlee C & O'Reilly DS. A routine method for the simultaneous measurement of retinol, alpha-tocopherol and five carotenoids in human plasma by reverse phase HPLC. Clin Chim Acta 1998;270:85-100.

Tapiero H, Townsend DM & Tew KD. The role of carotenoids in the prevention of human pathologies. Biomed Pharm 2004;58:100.

Tavani A, Gallus S, Negri E, Parpinel M & La Vecchia C. Dietary intake of carotenoids and retinol and the risk of acute myocardial infarction in Italy. Free Radic Res 2006;40:659-664.

Thurnham DI, Smith E & Flora PS. Concurrent liquid-chromatographic assay of retinol, alpha-tocopherol, beta-carotene, alpha-carotene, lycopene, and beta-cryptoxanthin in plasma, with tocopherol acetate as internal standard. Clin Chem 1988;34:377-381.

Tswett M. Concerning the macro- and micro-chemical composition of carotenes. Ber Dtsch Bot Ges 1911;29:630-636.

Underwood BA. Vitamin A deficiency disorders: international efforts to control a preventable "pox". J Nutr 2004;134:231S-236S.

Wachenroder H. Concerning the oil extracted from carrot roots, carotene, carotene sugars and components of carrot juice. Geiger's Magaz Pharm 1831;33:144-172.

Wang T, Yu WG & Powell WS. Formation of monohydroxy derivatives of arachidonic acid, linoleic acid, and oleic acid during oxidation of low density lipoprotein by copper ions and endothelial cells. J Lipid Res 1992;33:525-537.

Wang Y, Xu X, van Lieshout M, West CE, Lugtenburg J, Verhoeven MA, Creemers AF, Muhilal & van Breemen RB. A liquid chromatography-mass spectrometry

method for the quantification of bioavailability and bioconversion of beta-carotene to retinol in humans. Anal Chem 2000;72:4999-5003.

Visioli F, Riso P, Grande S, Galli C & Porrini M. Protective activity of tomato products on in vivo markers of lipid oxidation. Eur J Nutr 2003;42:201-206.

Wilson R, Smith R, Wilson P, Shepherd MJ, Riemersma RA. Quantitative gas chromatographymass spectrometry isomer-specific measurement of hydroxy fatty acids in biological samples and food as a marker of lipid peroxidation. Anal Biochem 1997;248:76-85.

Witztum JL. The oxidation hypothesis of atherosclerosis. Lancet 1994;344:793-795.

Vogt TM, Mayne ST, Graubard BI, Swanson CA, Sowell AL, Schoenberg JB, Swanson GM, Greenberg RS, Hoover RN, Hayes RB & Ziegler RG. Serum lycopene, other serum carotenoids, and risk of prostate cancer in US Blacks and Whites. Am J Epidemiol 2002;155:1023-1032.

Wolters M & Hahn A. LDL susceptibility to copper-induced oxidation after administration of a single dose of free or esterified beta-cryptoxanthin. Ann Nutr Metab 2004;48:163-168.

Woodall AA, Britton G & Jackson MJ. Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: relationship between carotenoid structure and protective ability. Biochim Biophys Acta 1997;1336:575-586.

World Cancer Research Fund/American Institute for Cancer Research (2007). Food, Nutrition, Physical Activity and the Prevention of Cancer: a Global Perspective. Washington DC: AICR2007, Available:http://www.dietandcancerreport.org.

Wu K, Erdman JW,Jr, Schwartz SJ, Platz EA, Leitzmann M, Clinton SK, DeGroff V, Willett WC & Giovannucci E. Plasma and dietary carotenoids, and the risk of prostate cancer: a nested case-control study. Cancer Epidemiol Biomarkers Prev 2004;13:260-269.

Ye Z & Song H. Antioxidant vitamins intake and the risk of coronary heart disease: meta-analysis of cohort studies. Eur J Cardiovasc Prev Rehabil 2008;15:26-34.

Yeum KJ & Russell RM. Carotenoid bioavailability and bioconversion. Annu Rev Nutr 2002;22:483-504.

Yhtyneet Medix laboratoriot. Laboratoriokäsikirja 2009-2010. Available: http://www.yhtyneetlaboratoriot.fi/.

Ylönen K, Alfthan G, Groop L, Saloranta C, Aro A & Virtanen SM. Dietary intakes and plasma concentrations of carotenoids and tocopherols in relation to glucose metabolism in subjects at high risk of type 2 diabetes: the Botnia Dietary Study. Am J Clin Nutr 2003;77:1434-1441.

Yonekura L & Nagao A. Intestinal absorption of dietary carotenoids. Mol Nutr Food Res 2007;51:107-115.

Young AJ & Lowe GM. Antioxidant and prooxidant properties of carotenoids. Arch Biochem Biophys 2001;385:20-27.

Zhang J, Dhakal I, Stone A, Ning B, Greene G, Lang NP & Kadlubar FF. Plasma carotenoids and prostate cancer: a population-based case-control study in Arkansas. Nutr Cancer 2007;59:46-53.

Zhao X, Aldini G, Johnson EJ, Rasmussen H, Kraemer K, Woolf H, Musaeus N, Krinsky NI, Russell RM & Yeum KJ. Modification of lymphocyte DNA damage by carotenoid supplementation in postmenopausal women. Am J of Clin Nutr 2006;83:163-169.

Jouni Karppi

Measurement of Carotenoids and Their Role in Lipid Oxidation and Cancer

Carotenoids are colourful compounds, present in fruits and vegetables, synthesised by plants and micro-organisms. Carotenoids act as antioxidants and possibly decrease in vivo lipid oxidation. Lipid oxidation is known to be a risk factor for development of atherosclerosis. Carotenoids are thought to be responsible for the beneficial properties of fruits and vegetables in preventing human diseases, including cardiovascular diseases and cancer.
In this study, we observed that serum/plasma carotenoids may decrease lipidoxidation in vivo. In addition, high serum concentrations of lycopene may decrease the risk of cancer in middle-aged Finnish men.

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