Heinrich-Heine Universität Düsseldorf
Institut für Biochemie und Molekularbiologie I

Oxidants and Antioxidants in Human Health. Nutritional Intervention Study Based on the Campaign "5 a Day"

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Ph.D Dissertation

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Chapter 1

Introduction

1.1 Oxidants and Oxidative Stress

Aerobic organisms utilize oxidative catabolism as a highly effective method of extracting energy from food molecules [1]. The process of oxidative phosphorylation results in a major release of metabolic energy. In the process NADH and $FADH_2$ transfer the electrons gained (through the citric acid cycle) to molecular oxygen producing heat and ATP. However, partial reduction of the oxygen molecule can occur at the same time and reactive free radicals ($O_2^{\bullet -}$) are constantly generated. Free radicals are molecules or molecular fragments with an unpaired electron in its outermost shell of electrons. Free radicals can be formed via addition or release of an electron, or by homolytic cleavage of a covalent bond.

Univalent reduction of the oxygen molecule leads to the production of the superoxide radical $O_2^{\bullet -}$, which belongs to the group of reactive oxygen species (ROS). ROS are reactive forms of oxygen, radical and non radical (Table 1.1).

$O_2^{\bullet -}$ exists in equilibrium with its conjugated acid, the hydroperoxyl radical ($HOO^{\bullet}$), which dismutates to hydrogen peroxide [2], [3]. The hydroxyl radical ($OH^{\bullet}$) can be formed from the reaction of hydrogen peroxide (H_2O_2) with the superoxide radical ($O_2^{\bullet -}$) (A):

Table 1.1: Reactive oxygen species (ROS)

ROS			
Radicals		Non Radicals	
Superoxide	$O_2^{\bullet -}$	Hydrogen peroxide	H_2O_2
Hydroxyl	$OH^{\bullet}$	Hypochlorous acid	HOCl
Peroxyl	$RO_2^{\bullet}$	Hypobromous acid	HOBr
Alloxyl	$RO^{\bullet}$	Ozone	O_3
Hydroperoxyl	$HO_2^{\bullet}$	Singlet Oxygen	$^1O_2$

$$\text{A.)}\ H_2O_2 + O_2^{\bullet -} \longrightarrow O_2 + {}^{\bullet}OH + OH^-$$

Nitric oxide ($NO^{\bullet}$) is also a radical but it is neither a strong oxidizing nor a reducing agent. However, when produced in excess, or in addition to reactive oxygen species (ROS), nitric oxide undergoes a variety of reactions, which can produce much more reactive and potentially damaging free radicals (nitrative stress). Nitric oxide is produced within cells by three forms of nitric oxide synthase (NOS/ type I, II, and III: neuronal, inducible and endothelial). Nitric oxide reacts with superoxide to from a very potent oxidizing agent, peroxynitrite ($ONOO^-$) (B):

$$\text{B.)}\ NO^{\bullet} + O_2^{\bullet -} \longrightarrow ONOO^-$$

Often the assumption is made that ROS/NOS always mediate adverse reactions, but the human body also needs ROS/NOS as, for example, in their function in primary immune defense and for relaxation of smooth muscles in blood vessel-walls.

Sources of Reactive Oxygen Species (ROS) within Cells All cells produce ROS in the normal course of metabolism. The main sites for free radical generation are:

- The mitochondrial electron transport chain
- Peroxisomal fatty acid metabolism
- Microsomal P_{450} enzymes
- Phagocytic cells

Mitochondria are the major source of ROS [3]. Mitochondria may generate more than 85% of the ROS whithin a tissue, such as skeletal muscle [4]. Many tissues also contain a xanthine dehydrogenase/ xanthine oxidase enzyme system that generates superoxide when the cells are stressed [5]. Several other enzyme systems also generate ROS as part of their physiological function. Peroximal metabolism of fatty acids can lead to free radical formation and many of the intermediates in prostaglandine and leukotriene biosynthesis and metabolism are reactive oxygen species, which may contribute to the generation of oxidative damage. Most important, are the NAD(P)H oxidases located in the plasma membrane of neutrophils and various other cell types. NAD(P)H oxidase is responsible for the generation of radical species as part of the cellular response to invading organisms [3]. Numerous xenobiotics are metabolized to free radical species by normal cellular detoxification mechanisms such as the cytochrom P_{450} system [6], [3]. This may be a relatively minor component of the cellular sources of ROS in normal situations, but under specific conditions (e.g. cigarette smokers) the system may be an important source of oxidants in the body. In addition UV and ionizing radiation can generate ROS.

Oxidative Stress If the rate of ROS production exceeds existing antioxidants defense systems, *Oxidative Stress* "an imbalance between oxidants and antioxidants in favor of the former, potentially leading to damage" can arise [1], [2], [7].

In such a situation, a whole series of cellular responses can be triggered leading to further increase in the generation of ROS.

Excessive generation of ROS can wreak havoc within cells and tissues, inducing cellular damage that may ultimately lead to cell death [8].

In addition, oxidatively damaged cellular macromolecules trigger immune responses that may lead to disease.

Many common diseases are associated with oxidative stress [1]. Oxidative stress may not be the factor initiating disease, but its progression can be influenced significantly through resultant oxidative stress. Also, ROS can be produced through exposure to environmental oxidants, toxic agents, and heavy metals, which perturb the equilibrium between cellular antioxidants and oxidation reactions and interfere with normal biological functions.

A substantial body of information has built up to support the hypothesis of Harman [9], who already in 1956 postulated that oxidative damage is associated with the aging process and that this is due principally to the generation of oxidative species. The postulate that the aging process itself is a result of increasing oxidative damage and free radical generation, particularly involving the mitochondria, could explain the correlation that exists between the aging process and increased risk of degenerative diseases. Diseases of aging, including cancer, cardiovascular disease, dementia, cataract, type 2 diabetes and autoimmune diseases, have all been shown to be associated with oxidative stress. To counteract the oxidant effects and to restore homeostasis, cells must activate genes encoding regulatory transcription factors, antioxidant defense enzymes, and structural proteins [8], [10].

1.2 Antioxidants

As a result of the continuous production of ROS, various strategies of antioxidant defenses have evolved in aerobic cells. The antioxidant defense system is composed of:

- endogenous antioxidant compounds
- exogenous antioxidant compounds

The endogenous antioxidant system includes enzymatic (constitutive and inducible) and non-enzymatic antioxidants, which detoxify ROS. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) [8], and glutathione peroxidases (GPx) provide the first line of defense. SOD catalyzes the dismutation of the super oxide radical ($O_2^{\bullet -}$) to oxygen and peroxide, which is protonated to form hydrogen peroxide (H_2O_2):

$$O_2^{\bullet -} + O_2^{\bullet -} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

By removal of $O_2^{\bullet -}$, SOD inhibits the superoxide-driven Fenton chemistry, preventing the formation of ferrous ions:

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$
$$2\ O_2^{\bullet -} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$
$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + OH^{\bullet} + Fe^{3+}$$

Although H_2O_2 is a powerful oxidant, it is relatively unreactive towards most biologic substrates unless it is present in unphysiologically high concentrations. However, in the presence of ferrous ions, H_2O_2 readily reacts to form the highly reactive hydroxyl radical (OH^-). CAT converts H_2O_2 in water and oxygen (1) preventing the formation of OH^-. GPx reduces H_2O_2 and organic hydroperoxides using glutathione (GSH) as substrate (2):

1. $H_2O_2 + H_2O_2 + \xrightarrow{CAT} 2\ H_2O + O_2$
2. $2\ GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$

Indirect antioxidant functions are mediated by enzymes that restore endogenous antioxidant levels; e.g. glutathione (GSH) levels are restored via reduction of oxidized glutathione (GSSG) by glutathione reductase. Reactive products or xenobiotics, e.g. epoxides, can be detoxified by phase II detoxification enzymes such as glutathione-S-transferases to favor their excretion. Metal chelating plasma proteins (e.g. transferrin, ceruloplasmin and albumin) prevent the formation of ROS by controlling the levels of free iron or copper ions. These metal chelating proteins bind the redox active metals and limits the production of free radicals.

Exogenous antioxidant compounds in humans are delivered trough the diet. Fruit and vegetables provide a range of different compounds that possess antioxidant activities or have been suggested to scavenge the ROS based on their structural properties [11].

The most prominent dietary antioxidants are vitamin C, tocopherols (vitamin E), carotenoids and flavonoids [12]. Antioxidants have different solubilities, such as water-soluble ascorbate; lipid-soluble tocopherols and carotenoids, and intermediatory-soluble falvonoids, which are distributed across the phases of tissues, cellular macromolecular structures, cytosol or membranes [12], [2].

The protection against ROS provided by fruit and vegetable consumption could arise through an integrated reductive environment delivered by antioxidants of different solubility in each of the tissues, cellular and macromolecular phases [13].

The water-soluble vitamin C has a high reducing power and is able to scavenge a variety of ROS. Vitamin C is not synthesized in humans and must be thus acquired through the diet. Vitamin C also exhibits a number important physiological activities that are not related to its antioxidant properties.

Vitamin E (tocopherols), is a generic term for a series of naturally occurring tocopherols and tocotrienols (α, β, γ and δ-homologues having R-configuration at all three chiralic centers of the molecule). Among them, α-tocopherol displays the highest biological activity in vivo, which is not necessarily related to its antioxidant power. Vitamin E is generally accepted as the primary lipid-soluble antioxidant in the human, although other physiological activities have been described [14], [15].

At least 60 carotenoids occur in fruit and vegetables consumed by humans. Besides the pro-vitamin A carotenoids, α, and β-carotene and β-cryptoxanthin, non-provitamin A carotenoids such lycopene and the hydroxy-carotenoids (xantophylls) lutein and zeaxanthin are major carotenoids present in the daily diet. Carotenoids are important not only for their pro-vitamin A activity, but also for a spectrum of other actions in biological systems [16].

1.2.1 Carotenoids: an Overview

Occurrence Carotenoids are usually yellow-red isoprenoid polyene pigments widely distributed in nature. Some 600 carotenoids have been identified. They are found in plants, algae, fungi, bacteria, animals and humans.

Their major role in plants is light harvesting as auxiliary components and quenching of excited states that might be formed during photosynthesis. Their presence in green plants is obscured by chlorophyll. Animals and humans cannot synthesize carotenoids *de novo*. However, carotenoids are present in human tissues and plasma as a result of dietary intake. Because of their lipophilic character, carotenoids are found associated with lipid structures like adipose tissue, membranes and LDL-cholesterol. Carotenoids are not evenly distributed in human tissues and organs. For instance, the *macula lutea* of the eye contains lutein and zeaxanthin exclusively and lycopene accumulates preferentially in testes. Rich sources of dietary carotenoids are strongly colored fruits and vegetables like tomatoes and carrots and

Table 1.2: Sources for main carotenoids

Lutein + Zeaxanthin	Lycopene	α-Carotene	β-Carotene	β-Cryptoxanthin
Kale	Tomato	Carrot	Carrot	Avocado
Spinach	Watermelon	Collard	Apricot	Orange
Broccoli	Pink grapefruit	Pumpkin	Mango	Papaya
Peas	Papaya	Cron	Kale	Passion fruit
Brussels sprout	Guava	Yellow paprika	Red paprika	Paprika
Collard	Roseship	Cloudberry	Spinach	Persimon
Lettuce			Broccoli	
Corn				
Egg yolk				

green, leafy vegetables like spinach and collard.

Major sources of selected carotenoids are presented in Table 1.2.

Structure Carotenoids are in their majority tetraterpenes formally composed of eight isoprene units. A typical example is β-carotene. Hydrocarbons are referred to as carotenes, such as lycopene, α-carotene, β-carotene (Fig. 1.1).

Oxygenated derivatives of carotenoids are called xanthophylls, such as lutein, zeaxanthin, β-cryptoxanthin and astaxanthin (Fig. 1.2).

Elements other than carbon, hydrogen, and oxygen are not directly attached to the carbon skeleton in naturally occurring carotenoids.

The most characteristic structural feature of a carotenoid is the conjugated polyene chain. The polyene chain represents a chromophore responsible for the characteristic color, going from colorless (phytoene, Fig. 1.3), to yellow (lutein, Fig. 1.2), orange (β-carotene, Fig. 1.1), red (lycopene, Fig. 1.1) and blue with an increasing number of conjugated double bonds.

The polyene chain is responsible for the general instability of carotenoids towards air oxidation, strong acids, oxidizing reagents, heat and light, necessitating particular precautions during isolation processes. Work with carotenoids must therefore

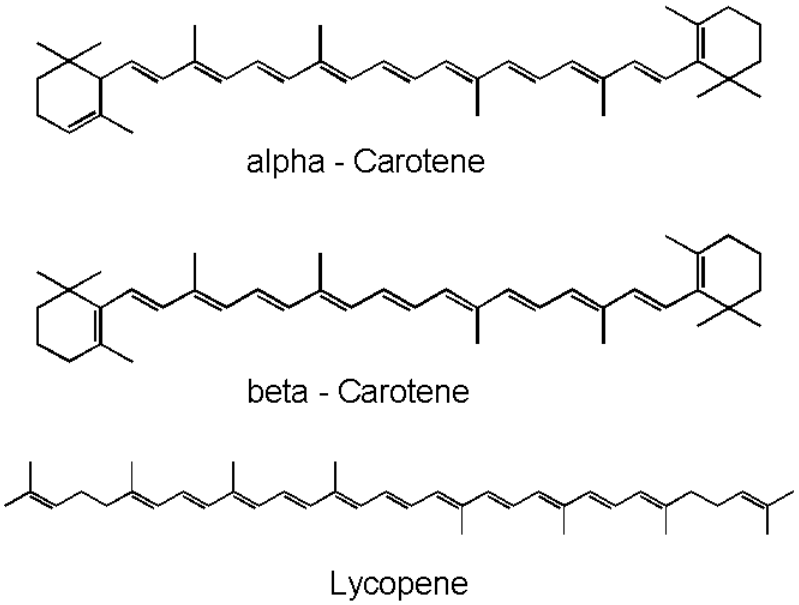

Figure 1.1: Structures of hydrocarbon carotenoids

take place under subdued light in an inert atmosphere (N_2) in the absence of strong acids and peroxides.

Of particular importance to the present study are lutein, lycopene, α-carotene, β-carotene, β-cryptoxanthin and zeaxanthin.

Bioavailability The bioavailability of carotenoids from raw fruits and vegetables is generally low but heat treatment of the food and concomitant intake of fat increase their bioavailability. At the same time, heat treatment may lead to loss of carotenoids and isomerisation, i.e., formation of *cis*-isomers. From dietary supplements carotenoid bioavailability is higher because the compounds are not associated with the plant matrix, and fat in the form of vegetable oil is part of the formulation. The factors that affect carotenoid bioavailability and bioconversion, (e.g. conversion into retinol) are collectively summed up in the memnotic **SLAMENGHI** [17], [18]:

species of carotenoids, molecular ***l***inkage, ***a***mount of carotenoids consumed with a meal, ***m***atrix in which carotenoids are found, ***e***ffectors of absorption and bioconversion, ***n***utrient status of the host, ***g***enetic factors, ***h***ost-related factors, mathe-

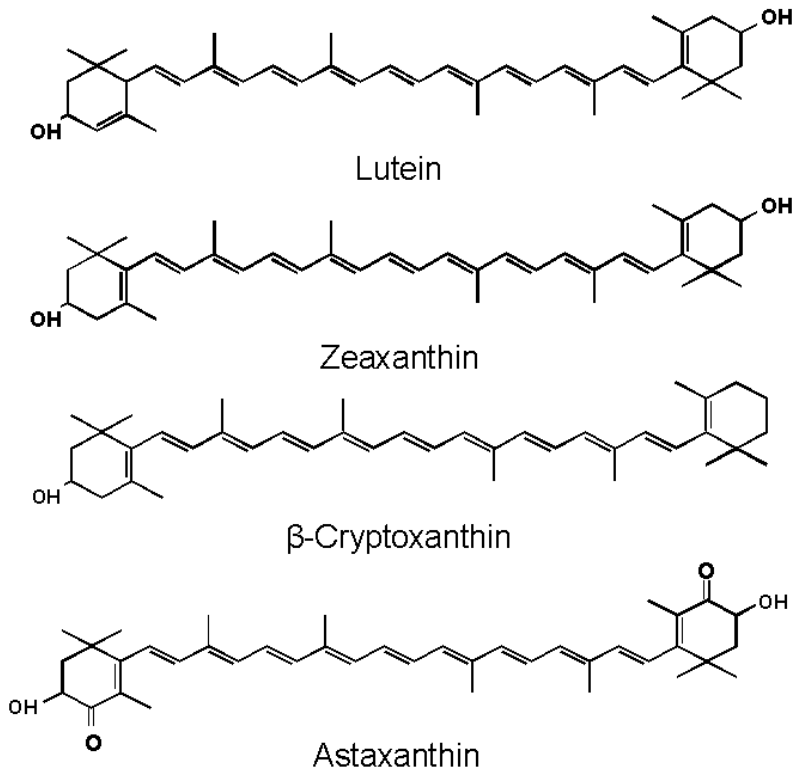

Figure 1.2: Structures of xanthophyll carotenoids

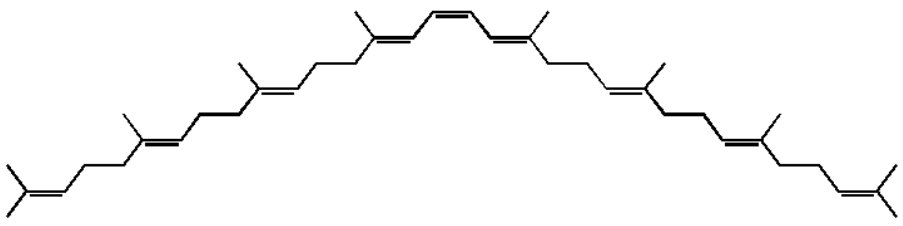

Figure 1.3: Phytoene structure

matical ***i***nteractions. Carotenoids themselves may also affect the bioavailability of other carotenoids, probably by competing for uptake, though such interactions are not yet clearly understood [19].

Because carotenoids are absorbed from the food, there are large individual variations in blood levels within a population (based on diet) [20]. There are of course also large variations from country to country depending on dietary patterns. A comparison of carotenoid intake in five Western countries (Ireland, UK, The Netherlands, France and Spain) showed large country-to-country variations both with respect to total carotenoid intake and the relative intake of different carotenoids [21]. There are not yet generally accepted dietary allowances for carotenoids in the USA or in Europe. The amount of carotenoids in the diet is difficult to estimate, partly because methods used for the establishment of food composition tables are not sufficiently specific or sensitive.

Health Effects Probably the best known biological effect of carotenoids is the provitamin A activity of some of them, in particular β-carotene that has twice the provitamin A activity of other provitamin A carotenoids. Some major dietary carotenoids have no provitamin A activity such as lycopene, lutein and zeaxanthin. Carotenoids are converted to vitamin A only as needed so that hypervitaminosis A has not been observed as a result of large doses of β-carotene.

Carotenoids are regarded as effective antioxidants *in vitro* and in some cases also pro-oxidants, but little evidence has been forthcoming to support the view that they act as pro-oxidants *in vivo* [22].

Antioxidant effects of carotenoids could explain why the consumption of a carotenoid-rich diet is associated with lower risk for oxidative stress related diseases, such as cardiovascular diseases or cancer. Many studies have shown an inverse association between intake of carotenoid-rich foods or serum levels of carotenoids and mortality. However, causality between carotenoids and mortality could not be established in these kinds of studies. Intervention trials with β-carotene alone or in combination with vitamins or other micronutrients failed to show a protective effect on cardiovascular disease, cancer or all-cause mortality [23]. In some cases, e.g., in heavy smokers, an increase in lung cancer incidence was observed [24], [25].

Participants receiving β-carotene alone, or in combination with vitamin E, had a significantly higher lung cancer incidence and higher mortality than subjects receiving placebo. It should be pointed out that the dosage of β-carotene in the ATBC study [24] was typically 10 times higher than what is found in an ordinary diet and that the bioavailability of β-carotene in the supplements used is higher than it is from food. Furthermore, intervention trials usually have a short time span compared to the development of cancer and cardiovascular disease, meaning that these studies have not established whether life-long supplementation of carotenoids at moderate physiological doses, rather than pharmacological ones, could be beneficial. Apart from β-carotene, other carotenoids have not been supplemented in large-scale intervention trials on humans. However, small scale clinical trials or pilot studies on lycopene supplementation have shown an inverse association between intake of lycopene and prostate cancer risk [26], [27],[28]. Rather than focusing on β-carotene or a particular carotenoid alone, a mixture of carotenoids as ingested with an antioxidant-rich diet may provide a broader range of beneficial effects and sound scientific evidence for carotenoid-related health claims.

1.3 Epidemiological Studies and the "5 a Day" Campaign

Vegetables, Fruits, and Cancer In the 1960s, the interest in dietary causes of human cancer was slowly revived by both the diffusion of the experimental model of laboratory chemical carcinogenesis and by migrant epidemiological studies suggesting that cancers are largely environmental in origin [29], [30], [31]. Specific hypotheses about diet and cancer emerged in the 1970s. Interest grew in the effects of fat, fiber, alcohol, and pickled foods. Insights into the cancer process increasingly suggested that diet might play a role in all stages of cancer development.

It was not until the late 1980s and early 1990s, however, that recognition of the role of plant foods in the diet began to consolidate. Summaries of the epidemiological literature specific to the relationship between vegetables and fruit and cancer were just emerging [32], [33], [34], [35], [36], [37], [38], [39], [40].

In one of the early review articles [40] it was shown that in 128 of 156 retrospective and prospective dietary studies calculating relative risk, a statistically significant inverse association was found between vegetable and fruit consumption and the occurrence of cancer at 13 different anatomical sites. These were cancers of the oral cavity, esophagus, pharynx, larynx, stomach, pancreas, colon, rectum, lung, bladder, endometrium, cervix, and ovary. It became clear that, of all the dietary factors postulated to be related to cancer, the evidence was most consistent for an inverse association between the risk of cancer and vegetable and fruit consumption.

Fruit and Vegetable Intake Based on the analysis of the epidemiological data available, a daily intake of at least 400 g of fruit and vegetables has been recommended by the WHO. More recently, the figure of 400 g/day has been supported following analysis of 250 observational studies, both case-control and prospective [41].

Studies on the median intake of fruit and vegetables in ten European countries (Table 1.3) have shown that there is a considerable number of consumers failing this target. The problem seems most acute with vegetable intake, where fivefold variations in intake are observed between Northern and Southern Europe [42]. The results obtained from one of the studies are given in (Table 1.3).

It has been calculated that over half of the EU member states have a mean intake level that is about 70% below the WHO target - i.e. intake is less than 275 g/day [43]. Even in countires such as Greece that has a relatively high mean consumption (511 g/day), a substantial proportion of the population (37%) falls below this target. In many European countries, there is a sharp decline in intake in the lower socio-economic groups. There is relatively little information available allowing to translate dietary intakes, which are always prone to large errors, into more meaningful data on adequacy of intake. Nonetheless, it seems clear that there are substantial numbers of individuals whose consumption of these foods falls far short of recommendations. Exhorting the population to consume more fruits and vegetables within a controlled program emphasizing the beneficial effects of a micronutrient-rich diet in mitigating oxidative stress related diseases might be effective in preventing such.

Table 1.3: Median intake of fruits and vegetables in Europe [42]

Country	Fruits (g/person/day)		Vegetables (g/person/day)	
	Male	Female	Male	Female
Greece	480	350	500	520
Italy	310	240	360	270
Portugal	220	170	330	240
Spain	260	190	265	200
France	190	140	260	200
Belgium	300	225	260	200
Switzerland	260	190	180	140
UK	180	130	180	140
Denmark	150	110	160	120
Germany	210	160	160	120
Netherlands	350	240	150	120
Austria	350	250	150	110
Ireland	130	90	150	110
Finland	200	150	130	100
Sweden	210	160	130	90
Norway	250	180	120	90
Iceland	190	140	80	60

5 a Day Campaign The "5 A Day for Better Health Program" (5 A Day) [44], which was initiated in 1991 in the U.S.A., was a large-scale, public/private partnership between the vegetable and fruit industry and the U.S. Government. Its goal since then has been to increase the average pro capita consumption of vegetables and fruits in the United States to five or more servings every day. The long-term purpose is to help decrease the incidence of cancer and other chronic diseases through dietary improvements. The specific program objectives are to increase public awareness of the importance of eating five or more servings of vegetables and fruit every day and to provide consumers with specific information about how to incorporate more servings of these foods into their daily eating patterns.

The campaign was introduced in Germany in May 2000. Nutritionists as well as

doctors have been the major supporters for the campaign lobbying within governmental and non-governmental institutions for the acceptance of the campaign. The web-site www.5amtag.de [44] provides information about the relationship between degenerative diseases and fruit and vegetable intake for the German population. The "5 a Day" campaign has also spread around the world. Among the countries promoting the campaign are: Canada, Mexico, Spain, France, U.K., Sweden, The Netherlands, Hungary, Japan, Australia and South Africa to mention some among the 20 countries which have joined the world wide program [45], [44].

1.4 Methods for Assessing Oxidative Stress

Observational epidemiological studies clearly show a correlation between the increased consumption of antioxidant-rich foods and a decreased risk of several oxidative stress related diseases [46].

The steady state balance between pro-oxidants and antioxidants in the human organism may be disturbed in case of depletion of the antioxidants, which may occur endogenously or as a consequence of a diminished dietary intake. The disequilibrium between free radicals and scavenging compounds, however, may be also due to an increase in the pro-oxidant load related to habits (such as smoking) or diseases. Because of apparent importance of ROS as toxins, mediators, and modulators of degenerative diseases, assessing oxidative stress might be useful to take preventive measures. ROS are, however, very short lived molecules and thus technically difficult to measure. One cannot ethically biopsy colon, prostate or brain in order to assess tissue damage. Therefore, the existing disbalance between pro-oxidants and antioxidants may be assessed by analysis of the diet-related antioxidant profile with the aim of having an insight in the ongoing condition of oxidative stress in human diseases [20].

How to Assess/Quantify Health Benefits from Antioxidants in Humans? A double-blind, placebo-controlled intervention trial in a sufficiently large population group remains the ultimate proof to demonstrate health benefits of antioxidants to lower disease risk. Traditionally, observational epidemiological studies on disease

risk were focused on clinical outcome with hard clinical endpoints. Such studies are indispensable, but they are time-and money consuming and may have intrinsic limitations in terms of rigorously controlling for possible confounders. The latter are usually multiple when studying oxidative stress in humans, because smoking habit, alcohol consumption, lipid profile, physical activity, antioxidant/iron supplementation, drug consumption - only to mention the major factors - throughly influence the oxidant/antioxidant balance of the organism.

A promising approach to strengthen epidemiological studies is the use of ***biomarkers***. The biomarker concept was discussed by ILSI FUFOSE Concerted Action in 1998 [47], [48].

A biomarker can be defined as an indicator on a biochemical, genetic or cellular level, reflecting exposure to a compound, susceptibility for a disease or the health status of a subject. A biomarker of oxidative stress reflects radical burden, (susceptibility of) oxidative damage, and oxidative stress mediated disease, or health status. For example, if we assume that direct damage to DNA by ROS contributes significantly to age-related development of cancer, then agents that decrease such damage should decrease the risk of cancer development. The steady-state level of oxidative DNA damage in human tissues is then a surrogate marker (biomarker) for later cancer development [49] [50]. In the physiological evaluation of a biomarker, it is important that there is a well established relationship between the response of the biomarker and the effect monitored. Ideally, the biomarker is the only target in the etiology. However, minor pathways may also provide suitable biomarkers. In the casual pathway of disease occurrence one might distinguish biomarkers of exposure (dietary intake), biomarkers of biological response and of (subclinical) disease and biomarkers of susceptibility (Fig. 1.4).

For example, blood levels of carotenoids (exposure marker) may be studied in relation to resistance of LDL to oxidation (a biological response/target function marker) or to carotid artery wall thickness (a disease/intermediate endpoint marker), in subjects with familial hypercholesterolaemia, or specific genotype (both susceptibility markers).

When studying specific diseases in humans, the measurement of the major plasma antioxidants (α-tocopherol, vitamin C and carotenoids) instead of all scavenging

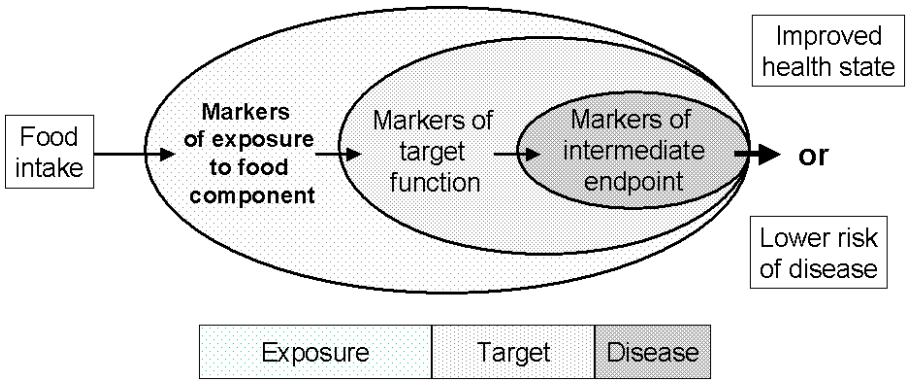

Figure 1.4: Classification of biomarkers related to the effect of antioxidants. Modified from ILSI Europe, 1999 [47]

molecules might be sufficient, but it is not recommended to study only one single antioxidant or the so-called total antioxidant status, since the levels may be strongly affected by dietary habits [20]. Therefore, biomarkers of exposure should accurately reflect relevant dietary intake or body status, while the "early disease" markers should have predictive value for the hard end-point. A further level of analysis would be the antioxidant profile in an afflicted target tissue, which might constitute a better indicator of disease-related oxidative stress than the plasma profile that integrates the patterns provided by all organs of the organism. An effective nutritional strategy will require knowledge of the type of antioxidants in the diet, their food sources, bioavailability and required levels of intake for protective effects. Before starting an intervention trial one should select the relevant markers and include a well-characterized study population. In the present intervention study, plasma levels of vitamins and antioxidants including six carotenoids were chosen as markers of exposure to be correlated with markers of oxidative stress (markers of biological response) in in a healthy working population. The selected study population was managed in the frame of the world wide campaign of "5 a Day".

1.5 Liposomes

Liposomes are vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids). They form spontaneously when lipids are dispersed in aqueous media, giving rise to a population of vesicles which may range in size from tens of nanometers to tens of microns in diameter. They can be constructed so that they entrap quantities of materials both within their aqueous compartment and within the membrane. The value of liposomes as model membrane systems derives from the fact that liposomes can be constructed of natural constituents in a way that the liposome membrane forms a bilayer structure, which is in principal identical to the lipid portion of natural cell membranes. The similarity between liposome and natural membranes can be increased by extensive chemical modification of the liposome membrane, and may be exploited in areas such as drug targeting, immune modulation or in vitro modeling of cell membranes challenged by free radical attack [51], [52]. For modeling oxidative stress on biological membranes, liposomes can be constructed having antioxidants anchored to the membrane, and thus inhibiting or delaying lipid peroxidation initiated by free radicals [53]. Damage to biological membranes by ROS using liposomes as model, can only be assessed indirectly by measuring lipid peroxidation products, such as malondialdehyde (MDA) or conjugated dienes [54]. Figure 1.5 shows schematically lipid peroxidation in liposomes.

A free radical ($X^{\bullet}$) initiates the lipid peroxidation process by attacking the liposome's membrane, extracting a hydrogen atom from a polyunsaturated fatty acid and forming a lipid radical (a). The lipid radical reacts with oxygen forming a lipid-peroxyl radical (b), thereby propagating the chain reaction by extracting another hydrogen atom from a adjacent polyunsaturated fatty acid chain (c). In the present work, unilamellar liposomes where used to investigate possible synergistic antioxidant effects of membrane-anchored carotenoids in inhibiting lipid peroxidation.

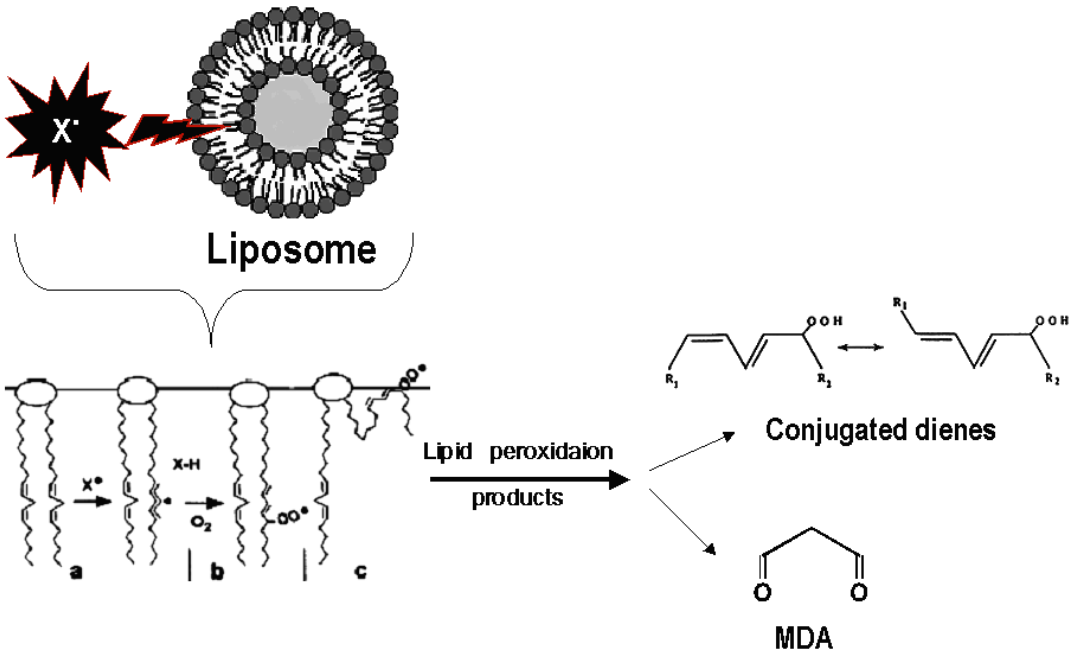

Figure 1.5: Lipid peroxidation process using liposomes as membrane model.

1.6 Total Equivalent Antioxidant Capacity, TEAC

Methods that have been developed for the measurement of the antioxidant activity of fluids are all essentially inhibition methods: a free radical species is generated, there is an end point by which the presence of the radical is detected, and the antioxidant activity of the added sample inhibits the process by scavenging the free radical:

Generate radical $\longrightarrow$ add antioxidant $\longrightarrow$ observe inhibition of endpoint

Methods vary greatly as to the radical that is generated, the reproducibility of the generation process, and the end point that is observed. An important consideration that had been overlooked in the various radical-generating based assays was the reliability and practicability of the method, e.g., earlier methods were based on inhibition of spontaneous tissue autoxidiation by antioxdiants. A method based on the effect of antioxidants on the absorbance of the radical cation of 2,2´-azinobis(3-ethylbenyothiazoline 6-sulfonate) was developed [55], [56]. This compound, trivially

known as ABTS (correctly, $ABTS^{\bullet+}$), has a characteristic long-wavelength absorption spectrum showing maxima at 660, 734, and 820 nm (Figure 1.6).

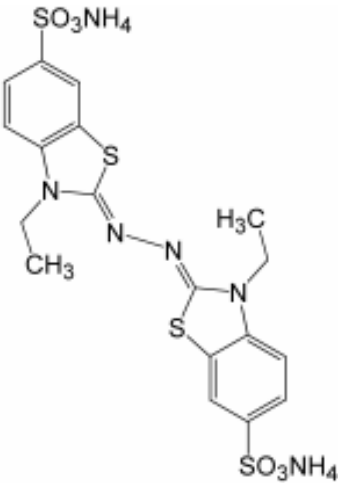

Figure 1.6: ABTS, 2,2' - azinobis(3-ethylbenzothiazoline 6-sulfonate)

The Trolox Equivalent Antioxidant Capacity, TEAC assay, is an improved assay [57], based on the scavenging of the $ABTS^{\bullet+}$, converting it into a colorless product. The degree of this decolorization reflects the amount of $ABTS^{\bullet+}$ that has been scavenged and is usually determined at 734 nm. The TEAC value (or Trolox equivalents) is assigned by comparing the scavenging capacity of an antioxidant to that of Trolox (Figure 1.7), which by convention is always 1.

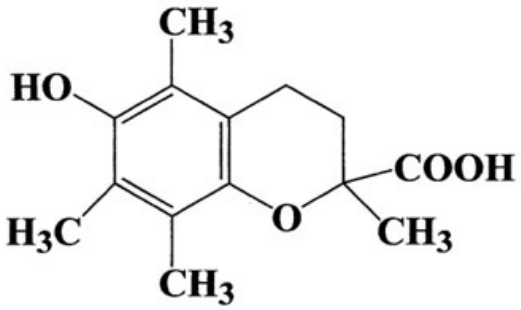

Figure 1.7: Trolox, reference compound.

Hydrophilic as well as lipophilic antioxidants or antioxidant mixtures can be compared by assigning them a TEAC value. Since the TEAC values are relative,

they cannot be compared to other assays which also measure antioxidant capacity, despite the fact that most of them use also Trolox as reference compound.

1.7 Aim of the Thesis

The aim of the present thesis work was to carry out an intervention study on a working population within the world wide campaign of "5 a Day", to test whether a counseled diet of five portions of fruits and vegetables a day, would increase the levels of antioxidants/micronutrients in blood and consequently influence the levels of the biomarkers for heart diseases and oxidative stress.

Another aspect of the study was to evaluate the volunteers compliance according to the study design in order to identify micronutrients, in special carotenoids, showing a significant increase after the intervention and attributing this increase to a particular dietary item consumed, that is: to correlate the increase of blood micronutrient levels (their accumulation) with the consumption of a certain fruit or vegetable type. Furthermore, carotenoids showing a significant increase, should be further tested for possible synergistic effects as single compounds or in mixtures in a bilayer compartment model system using liposomes. Also carotenoid mixtures were to be evaluated for synergism and antioxidant potency in a single compartment model using homogeneous solutions.

Chapter 2

Subjects and Methods

2.1 Intervention Study Design

The intervention study ran under the name "Take 5 Portions a Day of Fruits and Vegetables to Reduce the Risk of Cardiovascular Diseases" *("5 am Tag - Obst und Gemüse" zur Verminderung des Risikos kardiovaskulärer Erkrankungen)* was carried out during 3 months with a starting number of 129 volunteers. A waiting list of 52 subjects who could not take part in the study due to availability reasons was created in addition. Due to "drop outs" during the course of the study the final number of participants included for the final analysis was 112 individuals. The study was not randomized and without a control group. The recruitment of the volunteers was by means of electronic and postal advertising within clinical departments and administrative offices of the UKD, Universitäts Klinikum Düsseldorf. It was an open invitation for participation, hence no specific restrictions were imposed. The volunteers were distributed into 6 groups, each one of about 20 individuals. Small group size was chosen in order to address the participants directly and guarantee efficient teaching. Each group was offered an identical intervention plan consisting of 4 (T0, T1, T2, T3) 90 min. group meetings on a monthly basis for a period of 3 months (twelve weeks), hence having the meetings at begin of the study (T0), and

then after four (T1), eight (T2) and twelve (T3) weeks. In the sessions, guided by a nutritionist and a physician, participants were informed about the health effects of the "Five a Day" program in order to motivate the volunteers to change their nutritional habits accordingly. "Questions and Answers" workshops about the study were included (see nutrition quiz).

Preparation of the Study A protocol was created in detail to formalize and standardize the procedures for data collection such as filling out of standardized forms and dietary questionnaires (see "Food Frequency Questionnaire" on section 2.2.2). Storing, transport and analysis of blood samples as well as the medical tutoring of volunteers was included in the protocol. It was also established how the volunteers will be informed about the study, the concept of taking "5 a Day" and the content of the work shops and group meetings.

Location of the Study The study was performed at the UKD, the analysis of the blood samples was carried out at the Institute of Biochemistry and Molecular Biology I. Both facilities are located on the campus of the Heinrich-Heine University of Düsseldorf.

2.2 Subjects

For the execution of the intervention study applicants had to sign a written consent on their agreement of how the study would be carried out and how they as volunteers would be involved in it. In the written consent the volunteers explicitly state that they have been informed by the study director (a medical doctor from the Institute of Biochemistry and Molecular Biology I, of the Düsseldorf Heinrich-Heine University) about the conditions of the study. Volunteers who signed up could leave the study at any point without further justification or explanation. They were aware that the study had as an aim, the investigation of the influence of the consumption of five portions of fruits and vegetables on specific blood parameters as well as on their health status determined by the analysis of their blood samples. Furtheron the volunteers freely accepted to take part in all four group meetings and fill out

of the Food Frequency Questionnaires (seven in total) and donate four blood samples. The volunteers gave their consent to entitle the Institute of Biochemistry and Molecular Biology I, the ownership of the data collected.

2.2.1 Recruitment of Volunteers

The recruiting of individuals started on January 2^{nd} 2004, two months prior to study starting point. The purpose of the study and a small informative introduction was distributed per e-mail and regular mail among the staff of the UKD. A cost free participation was clarified. The interested candidates were invited for a further presentation of the program. An information flier was given to the interested individuals in which they were informed about the aims and goals of the study. Scientific background as well as the Europe-wide action of "5 a Day" to lower the risk of degenerative diseases were also mentioned. For encouragement, the advantages of taking part in this study were also listed in the flier. For the convenience of the participants a calendar with the exact dates of the group meetings was included (Table 2.1).

The demands of the study, such as blood drawing, attendance of the group meetings, etc., were also highlighted. The volunteers were informed about the length of the study, i.e. three months. They were reassured that the data collected during the study would serve only for scientific purposes and would be handled confidentially.

Group Meetings The meetings, arranged between 1^{st} of March and 2^{nd} of June 2004 (Table 2.1), were carried out in group sessions and workshops under the control of qualified staff. For every group meeting there was a 90 min. group session and a workshop scheduled.

At the group sessions participants were given detailed instructions about food preparation and coping with adversities in adhering to the new eating habits. The topics presented consisted of information regarding the world wide campaign of "Take 5 a Day" (www.5aday.org), its purpose and principles in scope of a diet rich in fruit and vegetables, the consequences related to a loss of micronutrients in the organism and the concept of bioavailability. Antioxidants, the concept of

"Oxidative Stress" and unhealthy habits like lack of exercise and smoking were highlighted within this framework.

The workshops serve as a means of motivation, counseling and attendance of individual questions regarding the study. Volunteers got practical advice about different techniques of food preparation and avoidance of vitamin loss through storage and handling of food. These basic concepts for a healthy diet and the necessary daily intake of vitamins and micronutrients was exemplified with the presentation of various nutrient rich fruits and vegetables which serve as a demonstration of how to achieve the 5 portions during the study accompanied with informational material, nutritional tables and nutrition tips.

Participants were also asked to self-complete a standardized Food Frequency Questionnaire (FFQ) slightly modified from Winkler and Döring [58] after every session and at home between two consecutive sessions, in order to monitor, in addition to the intake of other food items, the intake of specific fruit and vegetable categories over time more reliably.

At each of the four group sessions, blood samples (see 2.3.1) were collected, by assisting physicians, who also collected medical history and recorded height and body weight on the first and last session for the BMI calculation. Table 2.1 shows the time table of the group sessions held.

Table 2.1: Time table of group meetings between 1^{st} of March and 2^{nd} of June, 2004.

"5 a Day" Group meetings in 2004				
Group	T0	T1	T2	T3
1	March 1^{st}	March 29^{th}	April 26^{th}	May 24^{th}
2	March 2^{nd}	March 30^{th}	April 27^{th}	May 25^{th}
3	March 3^{rd}	March 31^{st}	April 28^{th}	May 26^{th}
4	March 3^{rd}	April 1^{st}	April 29^{th}	May 27^{th}
5	March 8^{th}	April 5^{th}	May 3^{rd}	June 2^{nd}
6	March 9^{th}	April 6^{th}	May 4^{th}	June 1^{st}

2.2.2 Collection of Socio-Economical Data

Socio-economical data were collected for the study to understand the social, demographical and laboural background of the volunteer. To acquire this information the base line questionnaire was divided into two parts. The first part contained questions about the volunteer´s personal data such as age, gender, educational background, work load and family status. This is usually referred to as demographical data. The second part was focused on questions related to the volunteers health and habits such as smoking, exercise, dietary habits, illness and regular medication, allergies and emotional state related to his/her job.

The base line data were used later to correct for possible confounders in the statistical analysis (see 2.3.6).

Body Mass Index (BMI) The BMI is the relationship between the individuals height and their body´s fat content. The BMI is independent from the size of the body, and is a standard method to compare individuals. The BMI is defined as:

$$BMI = bodyweight(kg)/bodyheight^2(m^2)$$

Based on the BMI Europeans are categorized as:

Normal weight	BMI between 18.5 and 24.9
Overweight	BMI between 25.0 and 29.9
Adipositas	BMI over 30 (Adipositas is graded from 1-3)
Pathological	BMI over 40

BMI was calculated at the start and the end of the intervention. Aim of measuring the BMI was to correlate a possible change with items determined in the food frequency questionnaire in order to observe the impact of the counseled nutrition on the participants body weight.

Food Frequency Questionnaire (FFQ) A way to control and follow the eating behavior of the participants was by recording their diet by self completing a standardized food frequency questionnaire (FFQ), of food items consumed (Fig.2.1).

By filling out the FFQ, it was possible to correlate later the increase of micronutrients levels to a specific food item consumed. In the FFQ the participants were asked about the type of food ingested in an approximate amount by ranking it as presented in Table 2.2.

Table 2.2: FFQ ranking table

Ranking	Frequency
1	many times daily
2	about once daily
3	many times during the week
4	about once a week
5	less than once a week
6	never

The items listed in the questionnaire are grouped according to the German Federal List of Food Classification, *(Bundesschlüssel für Lebensmittel)*. Items mareked as "other" were those food types which were not listed or did not belong to any of the groups listed in the FFQ, for example coffee. The original FFQ (German language) is presented in Figure 2.1. The translated FFQ is summarized in Table 2.3.

Table 2.3: Translation of the original Food Frequency Questionnaire, FFQ. Volunteers had to register their consumption of different fruit items in the past 14 days. During the intervention study, each volunteer provided seven of such FFQ.

Food consumption in the past 14 days	1	2	3	4	5	6
Meat (with no sausages)						
Sausages, ham						
Poultry						
Fish						
Potatoes						
Pasta						

...continuation

Food consumption in the past 14 days	1	2	3	4	5	6
Rice						
Raw salad or vegetables						
Leaf salad with dressing						
Raw salad						
Celery, beet, spinach (leafy vegetables)						
Green beans (peas)						
Egg-plant, cucumber, paprika, tomatoes, zucchini						
Corn						
Cauliflower, broccoli, cabbage, stem-savoy cabbage, sprouts						
Sauerkraut						
Fennel, leek, asparagus, onions						
Carrot, radish, beetroot, salsify, root vegetables						
Mushrooms						
Cooked vegetables						
Fresh fruits						
Blackberry, strawberry, raspberry, currant, huckleberry (berries)						
Grape						
Apple, pear, quince						
Apricot, cherry, mirabelle, plum, peach (stone-fruit)						
Banana						
Pineapple, kiwi, mango, maracuja (tropical fruits)						
Grapefruit, tangerine, orange, lemon (citric fruits)						
Raisins, dry fruits						
Fruit pure						
Chocolate, pralines						
Cake, pastry, cookies						
Other candies (lollipop, etc.)						
Salty finger food (peanuts, crisps, chips, etc.)						

...continuation

Food consumption in the past 14 days	1	2	3	4	5	6
White bread, toast bread						
Whole grain bread, brown bread, crisp bread						
Corn flakes, oat flakes/bran, granola						
Curd cheese, yogurt, soured milk						
Cheese						
Eggs						
Milk, buttermilk						
Fruit juices						
Multivitamin beverages						
Soft Drinks (Coca-Cola, lemonade, etc.)						
Mineral water, sparkling water						
Diet soft drinks						
Others						

2.2.3 Intervention

The intervention itself was the education of the participants in order to influence their eating habits in the daily consumption of five portions of fruits and vegetables or more. This was intended to be achieved by the informative talks held at the four group meetings. A summary of the meetings is presented below:

Start of the Study, T0. First group session and workshop. In the first session the volunteers were instructed and assisted in how to fill out the "Food Frequency Questionnaire". Socio economical information is collected from the participants containing items such as age, sex, weight, smoking habits, etc., (see 2.2.2). Volunteers were encouraged to ask questions; team work and active participation during the workshop was also encouraged.

Fragen zu Ihrer Ernährung >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>Zweite Messung

Fragebogen 5 am Tag G5.06.S3 Datum: 03.05.04

Wir möchten Sie bitten, zu schätzen, wie häufig Sie in den vergangenen 14 Tagen die in der Tabelle aufgeführten Nahrungsmittel zu sich genommen haben.
Bitte machen Sie für jedes von Ihnen verzehrte Lebensmittel ein Kreuz in die vorgesehene Spalte. Kreuzen Sie „nie" an, wenn Sie dieses Lebensmittel nicht zu sich nehmen.

Verzehr in den vergangenen 14 Tagen?	mehr-mals täglich	etwa 1 mal täglich	mehr-mals in der Woche	etwa 1mal der Woche	seltener als 1 mal in der Woche	nie
Fleisch (ohne Wurstwaren)				X		
Wurstwaren, Schinken			X			
Geflügel			X			
Fisch				X		
Kartoffeln			X			
Teigwaren				X		
Reis					X	
Salat oder Gemüse, roh zubereitet		X				
Blattsalat m. Dressing			X			
Rohkostsalat m. Dressing				X		
Bleichsellerie, Mangold, Spinat (Blattgemüse)				X		
Grüne Bohnen				X		
Aubergine, Gurke, Paprika, Tomate, Zucchini			X			
Gemüsemais			X			
Blumenkohl, Broccoli, Kohl, Kohlrabi, Rosenkohl, Wirsing			X			
Sauerkraut				X		
Fenchel, Lauch, Spargel, Zwiebel			X			
Möhre, Radieschen, Rettich, Rote Bete, Rüben, Sellerie, Schwarzwurzel		X				
Pilze			X			
Gemüse, gekocht			X			
Frisches Obst	X					
Brombeere, Erdbeere, Himbeere, Johannisbeere, Heidelbeere (Beerenobst)		X				

Figure 2.1: Original food frequency questionnaire (German)

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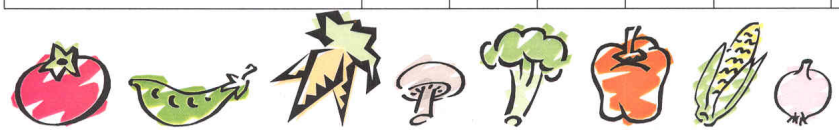
Verzehr in den vergangenen 14 Tagen?	mehr-mals täglich	etwa 1 mal täglich	mehr-mals in der Woche	etwa 1mal der Woche	seltener als 1 mal in der Woche	nie
Weintraube			X			
Apfel, Birne, Quitte (Kernobst)	X					
Aprikose, Kirsche, Mirabelle, Pflaume, Pfirsich... (Steinobst)			X			
Banane			X			
Ananas, Kiwi, Mango, Maracuja... (Südfrüchte)				X		
Grapefruit, Mandarine, Orange, Zitrone (Zitrusfrüchte)					X	
Rosinen, Trockenobst					X	
Kompotte						X
Schokolade, Pralinen				X		
Kuchen, Gebäck, Kekse				X		
Sonstige Süßwaren (Bonbons o.ä.)				X		
Salzige Knabbereien wie gesalzene Erdnüsse, Crisps etc.			X			
Weißbrot, Mischbrot, Toastbrot			X			
Vollkornbrot, Schwarzbrot, Knäckebrot			X			
Haferflocken, Müsli, Cornflakes				X		
Quark, Joghurt, Dickmilch		X				
Käse			X			
Eier				X		
Milch, einschl. Buttermilch		X				
Obstsäfte		X				
Multivitamingetränke			X			
sonstige Erfrischungsgetränke (Limonade, Cola-Getränke u.ä.)					X	
Mineralwasser	X					
Diätlimonaden, sonst. Diätgetränke						X
Sonstiges (bitte angeben):						
.....						
.....						



Vielen Dank fürs Ausfüllen!

2

Blood was collected from all the volunteers to determine base line of blood parameters. Body Mass Index (BMI) was calculated (see 2.2.2).

The attention of the participants to the study was drawn by presenting, in a simplified physiological perspective, the connection between antioxidants and degenerative diseases such as cardiovascular diseases (CVD), Alzheimer's Dementia, Type 2 Diabetes mellitus, and how micronutrients may act as preventing agents against the above mentioned diseases.

On the basis of nutritional information, an introduction on antioxidant and micronutrient rich nourishment was presented. The importance of a regular consumption of vitamins such as vitamin A, C, E, B6 were pointed out. Following the nutritional information about micronutrients and antioxidants, the counselor presented data about scientific evidence supporting the epidemiological findings of a negative correlation between a high consumption of fruits and vegetables and a low incidence of degenerative diseases, all this leading to the world campaign of "5 a Day". The concept of a portion was explained.

Examples of a possible composition of the "five portions" and exemplified presentations of certain nutrient rich fruit and vegetable sources were presented. Following the "rule of thumb", a handful of fruits and vegetables represents about a portion of 125 g. Examples for a single vegetable portion could be: 3 middle sized potatoes, 1 bowl of salad, $\frac{1}{2}$ plate of vegetables, $\frac{1}{2}$ plate containing cucumber, tomato, cauliflower; carrots; asparagus and paprika. Examples for a single fruit portion could be: 1 apple, 1 pear, 1 banana, $\frac{1}{2}$ pineapple, 1 bowl of cherries or grapes, $\frac{1}{4}$ of a watermelon.

With this information the basis for the intervention was paved. The volunteers were now aware of the importance of keeping a healthy diet, and the simple instructions of how to achieve it. The consequent meetings dealt with the topics mentioned above in more detail. An example of a nutritional quiz used during the workshops is presented in Figure 2.2.

T1 The topic of instruction and discussion of this session was entitled "Mental Fitness Through Nutrition". Among the information provided, the world wide population trend of aging and its accompanying degenerative diseases (stroke, Type 2

Quiz „5 am Tag"

Wieviel Gramm Obst und Gemüse entspricht "5 am Tag" etwa?
(Einfachauswahl – bitte ankreuzen)!

O 450 g
O 600 g
O 750 g

Welche Obst- und Gemüsesorten sind besonders reich an Carotinoiden?
(Mehrfachauswahl – bitte ankreuzen)!

O Broccoli
O Heidelbeeren
O Möhren
O Radieschen
O Mango

1 Portion von "5 am Tag" lässt sich ersetzen durch z.B.:
(Mehrfachauswahl – bitte ankreuzen)!

O 1 Glas Limonade
O 1 Glas Obstsaft
O 1 Glas Mineralwasser
O 1 Glas Milch
O 1 Glas Gemüsesaft

Ordnen Sie bitte zu: → Makronährstoffe / Mikronährstoffe?

Proteine	
Kohlenhydrate	Makronährstoffe
Carotinoide	
Vitamine	
Mineralstoffe	
Spurenelemente	Mikronährstoffe
Fette	

Figure 2.2: Original German language nutritional quiz. In this example volunteers were tested about practical advices regarding the "5 a Day" diet, being asked about portions, carotenoid rich foods, handling and storing of food, etc.

Lückentext: Bitte setzen Sie folgende Worte sinnvoll ein:
rot - ß-Carotin - Carotinoiden - gelb - Lycopin - Zeaxanthin - orange

Zu den über 600 bekannten ..... zählen u.a.
..... (Farbe:.....), ...
(Farbe:.....), und das bekannte..... (Farbe:.....).

Gesund ist bunt! Zählen Sie 5 möglichst farbstoffreiche Obst- und Gemüsesorten auf!

*
*
*
*
*

Der Vitamin-C-Verlust bei Tiefkühlware (Obst, Gemüse) ist durchschnittlich (Einfachauswahl - bitte ankreuzen)!
O niedrig
O mittel
O hoch

Hochdosierte Vitaminpräparate... (bitte markieren, ob richtig oder falsch)!

...sind immer sehr gesund.	richtig/falsch
...können wie ein Arzneimittel auch Nebenwirkungen haben.	richtig/falsch
...enthalten alles, was der Mensch an Nährstoffen braucht.	richtig/falsch

1 Tüte Kartoffelchips (200g) enthält ca.: (Einfachauswahl - bitte ankreuzen)!
O 20 g Fett
O 30 g Fett
O 70 g Fett

Ballaststoffe... (bitte markieren, ob richtig oder falsch)!

...können dazu beitragen, vor Darmkrebs zu schützen.	richtig/falsch
...sorgen für eine gute Verdauung.	richtig/falsch
...verursachen ein langanhaltendes Sättigungsgefühl.	richtig/falsch
...sind überflüssige Bestandteile der Ernährung.	richtig/falsch
...sind in Fleisch enthalten.	richtig/falsch

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Figure 2.3: ...continuation of the original German nutritional quiz.

diabetes, Alzheimer Dementia and heart failure, etc.) was highlighted. Mechanisms of antioxidant defense, contributing to the mental and physical fitness; the role of micronutrients, vitamins, minerals, fiber and secondary plant metabolites in fruits and vegetables in hampering age-related diseases was emphasized.

In this session the volunteers were instructed how to choose energy low and micronutrient rich nourishments and the way to prepare food in order to preserve as much as possible of the bioactive compounds and micronutrients. As in the first group session, a round of questions and answers was held. A "Food Frequency Questionnaire" was filled out. Blood samples were collected.

T2 During the last two meetings, the role of the protective actions of vitamins and micronutrients against brain damage and heart insufficiency was explained. A balanced healthy diet, based on the requirement of the macro and micronutrients´ seven components, protein, fat, carbohydrates, water, vitamins, minerals and trace elements was highlighted. The percentile distribution of macronutrients in the diet concerning energy requirements as well as the personal daily energy/calorie requirements and the "rule of thumb" of how to calculate it was also part of the central message of these session. Volunteers also learned about the concept of food´s *nutrient density*. The nutrient density gives information about a certain edible item concerning the ratio of a certain nutrient in comparison to its caloric value, calculated as: *the content of a specific nutrient in g/ kcal. in 100g food item.*

T3 At this last group session an additional questionnaire was filed out to re-check the socio-economical data. BMI is re-measured. A final "feed back" questionnaire was offered, where volunteers could express their personal opinion about the project. In general, volunteers noted that, the help, information and assistance provided through out the three months time of the intervention had been useful to follow the concept of "5 a Day" efficiently. They also felt they had been motivated all the time to follow the program till the end.

A summary of the activities in the whole intervention program is presented in Table 2.4.

Table 2.4: Intervention program. Between 1^{st} of March and 2^{nd} of June 2004.

Time points	T0		T1		T2		T3
days	0	15	30	45	60	75	90
Socio-Economical Data	x						x
Group meetings	x		x		x		x
Blood Sampling	x		x		x		x
BMI	x						x
FFQ	x	x	x	x	x	x	x

2.3 Methods

2.3.1 Blood Sampling

Blood sampling was in accordance to the principles outlined in the *Declaration of Helsinki* [59]. Blood plasma samples were collected in a heparinized tube and were immediately centrifuged at 3,850 g, for 15 min. at room temperature, and stored frozen at -80°C until analysis. Blood serum samples were collected without anticoagulant agent, left for 15 min. at room temperature and then centrifuged at 3,850 g, for 15 min. at room temperature, and stored frozen at -80°C until analysis. Blood sampling was carried out at 4 time points (T0, T1, T2, T3) for each individual at the time the group sessions were held (see table 2.4), collecting about 50 ml of blood plasma and serum, and analyzed for the parameters presented in Table 2.5, using HPLC, ELISA or commercial kits.

Blood plasma analysis of micronutrients and biomarkers of oxidative stress (see section 2.3.2) was carried out at the Institute of Biochemistry and Molecular Biology I, Heinrich-Heine University Düsseldorf. Clinical parameters were measured in blood serum in a clinical laboratory.

2.3.2 HPLC

High pressure liquid chromatography, HPLC, was used to analyze blood plasma levels of all micronutrients and the biomarkers homocysteine and MDA.

Table 2.5: Blood parameters analyzed. Micronutrients and biomarkers of oxidative stress were analyzed in blood plasma, clinical parameters in blood serum.

Clinical Parameters	Biomarkers of Oxidative Stress	Micronutrients
Uric Acid	Protein Carbonyls	Lutein
Cholesterol	MDA	Lycopene
HDL-Cholesterol	Homocysteine	α-Carotene
LDL-Cholesterol		β-Carotene
Triglycerides		β-Cryptoxantin
		Zeaxanthin
		Vitamin A (Retinol)
		Vitamin E (α-Tocopherol)
		Vitamin B6
		Vitamin C

Carotenoids Blood plasma levels of the carotenoids lutein, lycopene, α-carotene, β-carotene, β-cryptoxanthin and zeaxanthin were analyzed using a Suplex, pKb 100, 5μm particle column (Supelco, Bellafonte, PA) and detected at 450 nm [60], [61]. Retinol (Vitamin A) was analyzed using the same separation technique, detection was at 325 nm . All equipment used was from Merck-Hitachi: pump L-7100, UV-Vis. detector L-7420, L-4200, auto sampler L-2200 Elite LaChrom. The thermostat Jet-Stream 2-Plus was from Altmann- Analysentechnikvertriebs GmbH.

Figure 2.4 shows a typical HPLC chromatogram of a blood plasma sample analyzed for carotenoids with their respective retention times.

Tocopherols An UV/vis detector was connected in series to the system used for carotenoids´s analysis and set at 292 nm for quantification of α- and γ-tocopherol (vitamin E).

Vitamins Plasma levels of Vitamin C, Vitamin B6 were analyzed using a commercial kit of Chrom-Systems Instruments and Chemicals GmbH, Munich for HPLC. Columns and necessary reagents are provided with the kit. All the HPLC system was Merck-Hitachi: pump L-6200A, UV-detector L-4250, fluorescence detector L-

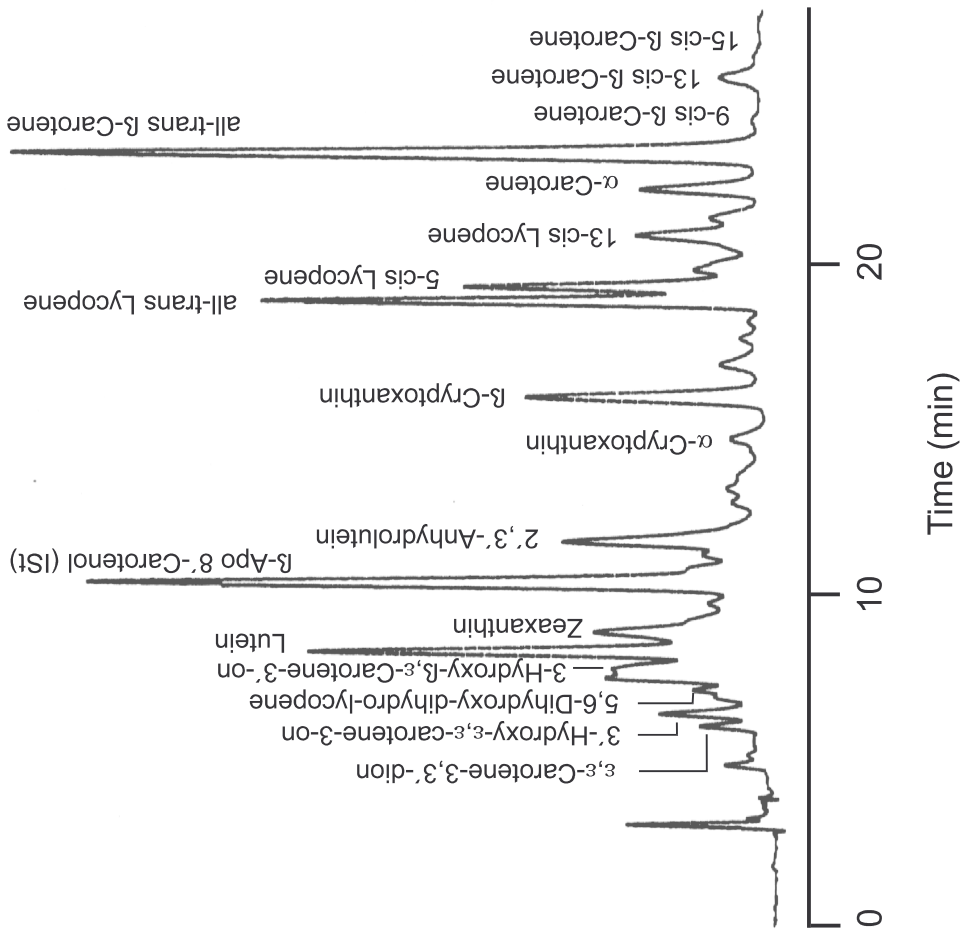

Figure 2.4: A typical HPLC chromatogram of a plasma sample.

7485, auto sampler L-7200. The thermostat Jet-Stream 2-Plus was from Altmann-Analysentechnikvertriebs GmbH. Data was collected from the HPLC system using the software program "D-7000 HSM, Chromatography Data Station Software".

Vitamin C For the analysis of plasma vitamin C, samples are cleaned up by protein precipitation contributing to stabilize vitamin C in the sample. Samples are analyzed using a commercial kit of Chrom-Systems Instruments and Chemicals GmbH, Munich for HPLC, in an isocratic HPLC system by UV-detection (245 nm) using an internal standard.

Vitamin B6 Vitamin B6 plasma level is determined based on the analysis of Vitamin B6 in its Pyridoxal-5-phosphate (PLP) form. Samples are freed from proteins followed by extraction and derivatization. Samples are analyzed using a commercial kit of Chrom-Systems Instruments and Chemicals GmbH, Munich for HPLC, in an isocratic HPLC system and detected fluorometrically (excitation 320 nm, emission 415 nm).

Biomarkers of Oxidative Stress Plasma levels of homocysteine and malondialdehyde (MDA), were analyzed using a commercial kit of Chrom-Systems Instruments and Chemicals GmbH, Munich, for HPLC. Columns and necessary reagents are provided with the kit. The HPLC system used was the same used for the vitamins´ analysis.

Homocysteine For total plasma homocysteine levels, protein bound homocysteine was released from its bound form. Samples are then freed from proteins, derivatized prior injection on an isocratic HPLC; detection was fluorometrically (excitation 385 nm, emission 515 nm).

Malondialdehyde, MDA For determination of MDA levels in plasma, samples are first freed from proteins by precipitation and then derivatized. The fluorophor formed is then detected fluorometrically (excitation 515 nm, emission 553 nm) using an isocratic HPLC system.

2.3.3 ELISA for Protein Carbonyls

For the measurement of protein oxidation, oxidated plasma Ig G was assessed. Protein carbonyls were measured using ELISA according to the method of Carty et al. [62] with slight modifications. Absorbance was measured spectrophotometrically at 490 nm. Carbonyl content was calculated from a standard curve and expressed as nmol carbonyl/ mg of Ig G.

Materials and Chemicals

96-well ELISA plates were from Nunc-Immuno Plate (F-96 442404). Desferal (D9533-1G), Tween-20 (P-8341) and o-Phenylediamine tablets (P-7288) were purchased from Sigma. 2,4 dinitrophenylhydrazine, DNPH (Sigma D-2630) was dissolved in 2 M HCl for a final concentration of 1mM. H_2O_2 (8.8 M) was from Merck. As primary antibody an anti-DNP antiserum, mouse Ig E (Sigma, D-8406) was used. The secondary antibody used was a HRP-labeled rat-antimouse IgE (Serotec: MCA 419P).

Buffer Solutions

Coating buffer A sodium carbonate solution of 50 mM at pH 9.2, was used as coating buffer, obtained by dissolving 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ in 1 liter H_2O.

Blocking and washing buffer Blocking and washing buffers were prepared by adding Tween-20 in 1% v/v and 0.05 % v/v in PBS respectively.

PBS PBS buffer of 0.15 M at pH 7.2, was obtained by dissolving 8.0 g $NaCl$, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 in one liter H_2O.

Citratephosphate buffer To obtain a 0.15 M of citrate phosphate buffer 21.0 g citric acid and 35.6 g Na_2HPO_4 were dissolved in one liter H_2O. The resulting solution's pH was corrected to pH 7.2.

Sample Clean-Up Of a fresh desferal stock solution 1 μl (100 mM) was added to 100 μl of blood plasma sample for a final concentration of 1mM. Desferal was added to the samples to chelate iron ions thus preventing samples from being oxidized. Samples were diluted (1:1) with 100 μl PBS. Plasma proteins were precipitated by addition of 100% saturated $(NH_4)_2SO_4$ for a final concentration of 45% (or a sample dilution of 9:11). After incubation at room temperature for about 30 min., samples were 3-4 times vortexed, and then centrifuged at 10 000 g for 15 minutes. After centrifugation the supernatant was discarded and the pellet washed in 1ml 45% $(NH_4)_2SO_4$, and recentrifuged as before. After discarding the supernatant, pellets were redissolved in PBS. If pellets were not used the same day for further analysis, they were kept frozen at -20°C. For Ig G protein determination samples were dissolved 1:10 in coating buffer. For ELISA carbonyl determination samples were dissolved 1:200 in coating buffer.

Protein Determination Blood plasma protein concentration was determined using the commercial kit, Bio-Rad, supplied with reagent A and B.[1] Samples were diluted approximately 1:10 with PBS buffer. 125 μl of Bio Rad reagent A was mixed with 25 μl of diluted blood plasma sample and subsequently mixed with 1 ml of Bio Rad reagent B. Samples were thoroughly vortexed and left at room temperature for 10 min. Absorption was measured at 750 nm. A calibration curve was prepared using the Bio Rad bovine serum albumin standard solution (Catalog number: 500-0007).

Assay Performance The estimated work load for 2 ELISA plates without dishwasher was 8-9 hours. Standards, antibodies and purified samples, were left slowly defrost at room temperature and then kept on ice until used. Samples and standards were diluted in coating buffer. Using a 96 well plate, 50 μl of samples and standards were pipetted in triplicates and incubated for 1 h at 37°C, in a cell culture incubator, a water bath incubator was avoided. After a plate was washed 3 times with washing buffer, derivatization was directly performed in the plate by adding 50 μl of DNPH into each well, incubation was for 1 h at 20-25°C.

[1]Commercial names given by the manufacturer to the reagents needed for the assay.

After derivatization, the plate was washed 3 times and 200 μl of blocking buffer was added into all wells in order to block any non-specific binding. Plates were then incubated for 1 h at 37°C or left over night at 4°C. After incubation plates were washed (3 times) followed by addition of 50 μl of the anti-DNP antiserum (primary antibody) diluted 1000-fold with blocking buffer. After addition of the primary antibody, plates were incubated for 2 h at 37°C and then washed (3 times) before adding the secondary antibody. 50 μl of the secondary antibody (HRP-labeled rat anti-mouse Ig E antibody) was added to the wells previously diluted 1:5000 with blocking buffer and incubated for 1 h at 37°C. Excess of unbound secondary antibody was removed by washing three times. The substrate for the HRP-labeled antibody was prepared by dissolving in 10 ml citrate buffer, 8 μl of H_2O_2 and 1 tablet of O-phenyldiamine. From the freshly prepared substrate 50 μl were added to the wells and incubated for 15 min. in the dark, at 20-25°C. Finally the reaction was terminated by addition of 50 μl of 2 M H_2SO_4. The orange-yellow reaction product was quantified at 490 nm using a plate reader.

Interruption of Assay In case the ELISA assay must be interrupted and continued next day it is advisably to stop the working up procedure at the following points:

- interruption after derivatization, the plates are washed and left on blocking buffer over night at 4°C.
- interruption after addition of either primary or secondary antibodies, plates are left on blocking buffer over night 4°C, then incubated next day at 37°C to carry on with the assay.
- interruption after antibody's addition and incubation at 37°C, plates were just left at 4°C and washed next day.

2.3.4 Liposomes

Chemicals L-α-Phosphatidylcholine from soybean (P-7443-1G) was purchased from Sigma. 2,2´-Azobis(2-amidinopropane) hydrochloride (AAPH), was purchased from Polysciences, Inc. (Cat. 08963). Zeaxanthin, lutein and lycopene were kindly

provided by BASF. Tetrahydrofuran (THF) free of BHT, was from Fluka (Cat. 8736). Sodium phosphate buffer (50 mM, pH 7.4) was prepared from Na_2HPO_4 (MW = 141.96) and NaH_2PO_4 (MW = 137.99), pH was set to 7.4 by correcting with $NaOH$ or H_3PO_4. For the determination of the liposome suspension's lipid concentration, the commercial kit "*Phospholipids B*", from Wako Chemicals, was used.

Extruder For the preparation of unilamellar liposomes from multilamellar ones, a LiposoFast extruder from Avestin, Inc. was used. The liposome emulsion was passed through a 400 nm polycarbonate membrane using two 500 μl Hamilton syringes. The extrusion device used in the present study is shown in figure 2.5

Unilamellar Liposome Preparation Lipid stock solutions were obtained by dissolving the lyophilized lipid dry powder in dichlor-methane (DCM), and stored in glass vials at -20°C. Due to DCM being readily volatile, stock concentrations had to be remeasured every time vials were opened (see lipid concentration determination on page 47).

Stock solutions of carotenoids were prepared in THF for an approximate concentration of 2 mM, and stored at -80°C. Working solutions of 2 μM were freshly prepared and checked spectrophotometrically prior to use. Table 2.6 shows the molar absorption coefficients of carotenoids used to calculate the respective concentrations. The carotenoid concentration in the liposomes was expressed as nmol carotenoid /mg lipid.

Table 2.6: Carotenoids with their respective absorption wavelength, molar absorption coefficient and solvent used for measurements.

Compound	λ (nm)	ϵ = molar absorption coefficient	Solvent
β-Carotene	450	139 000	Ethanol
Zeaxanthin	450	144 300	Ethanol
Lutein	445	144 800	Ethanol
Lycopene	472	184 575	Ethanol
Lycopene	487	180 600	Benzene

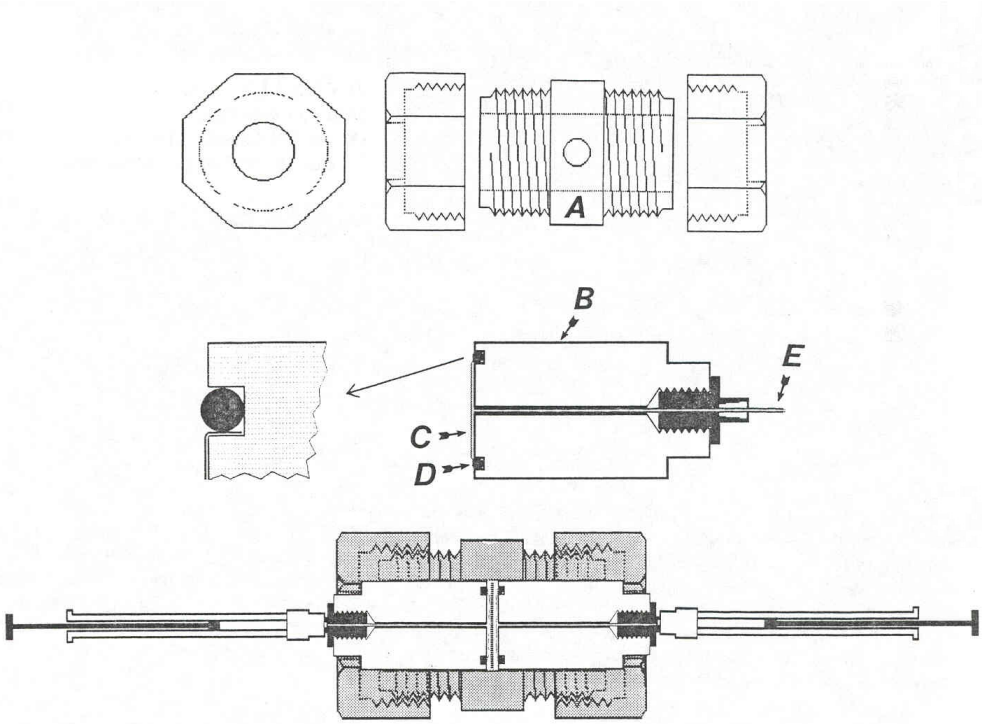

Figure 2.5: Extrusion device. The housing (A), with its two end caps, provides for confining and pressing together the membrane supports (B; two required) which have woven nylon screen (400 mesh is adequate) (C) on their surfaces to allow distribution of the suspension over the polycarbonate membrane (not shown in the figure) and 'O' rings (D) to retain the screen and prevent leakage of the sample. The stainless steel capillary (E) has a narrow bore to minimize hold-up volume. It is a tight press fit into the membrane support. The projecting end of the capillary is rounded and of appropriate length to fit into the teflon inner portion of a *Luer lock* Hamilton microsyringe. The black threaded portion is a female *Luer lock* bulkhead fitting to provide mechanical support for the syringes. A diameter for the supports (B) of between 0.5 and 1.5 inch is desirable and the polycarbonate membrane diameter is a few thousandths of an inch smaller in diameter than the supports. The size and proportions of the components are not critical. The housing(A) is fabricated from inert metal, the two membrane supports (B) and nylons (C) are made of plastic. To use the extruder, one support (B) is placed in the housing (A), from which one end cap has been removed, with the *Luer* connector projecting through the other end cap. The polycarbonate membrane is placed on the screen (C) surface of the membrane support. The second support is placed on the other surface of the polycarbonate membrane and the support is sealed against the filter by screwing on the second end cap. The assembled device is shown at the bottom of the figure.

Procedure Appropriate amounts of lipid and carotenoids were pipetted into a 250 ml round bottom flask and an additional 1 ml of DCM was pipetted to ensure a larger volume covering the flask surface. The flask was wrapped with aluminum foil to protect carotenoids from direct light. Lipids and carotenoids in the organic solvent were dried in a Rotavapor device using vacuum, rotating the flask slowly in a water bath at 45-50 °C for 10 min. The dry film was suspended in a nitrogen purged sodium phosphate buffer (100 mM, pH 7.4 at 25°C), for a final concentration of 10 mg lipid/ ml buffer. Flask was left rotate (no vacuum was needed at this step) for 5 min. at room temperature to ensure all lipid smear being suspended. If the lipid smear was not fully suspended, the flask was placed back in the water bath for 1 min. at 45-50°C with continuous rotation alternating it with room temperature rotation until all lipid was successfully brought into suspension. The liposomes obtained here were multilamellar and of various size. The flask was then flushed with nitrogen for 2 min. and placed in ultrasound bath for 10 min. at 25 °C. The lipid suspension containing flask was kept on ice in the dark until extrusion.

Extrusion A fresh 400-nm pore polycarbonate membrane was placed between the membrane supports which were then gently tighten by the end caps by hand. One Hamilton syringe was filled with the multilamellar liposome suspension and attached to the Luer locks. The suspension was passed gently back and forth 20 times through the extruder at room temperature avoiding direct light exposure. (see figure 2.6)

After extrusion, the suspension was diluted 1:10 with sodium phosphate buffer, and centrifuged at 20 000 rpm (68 600 RCF), using a SW 41 rotor in a Beckman L7-55 for 25 min. at 4°C. 1.5 ml of the upper part of supernatant containing the unilamellar liposomes was collected and stored in a cool, dark place at 4°C. Liposomes used were not older than 12 hours and checked by transmission electron microscopy (TEM) to prove that they were of unilamellar structure.

Transmission Electron Microscopy, TEM

Negative staining technique for transmission electron microscopy (TEM) was used to verify the liposomal structure (Fig. 2.7). Approximately 10 μl of the specimen solution (liposome sample diluted 1:100) was pipetted on a carbon-coated copper

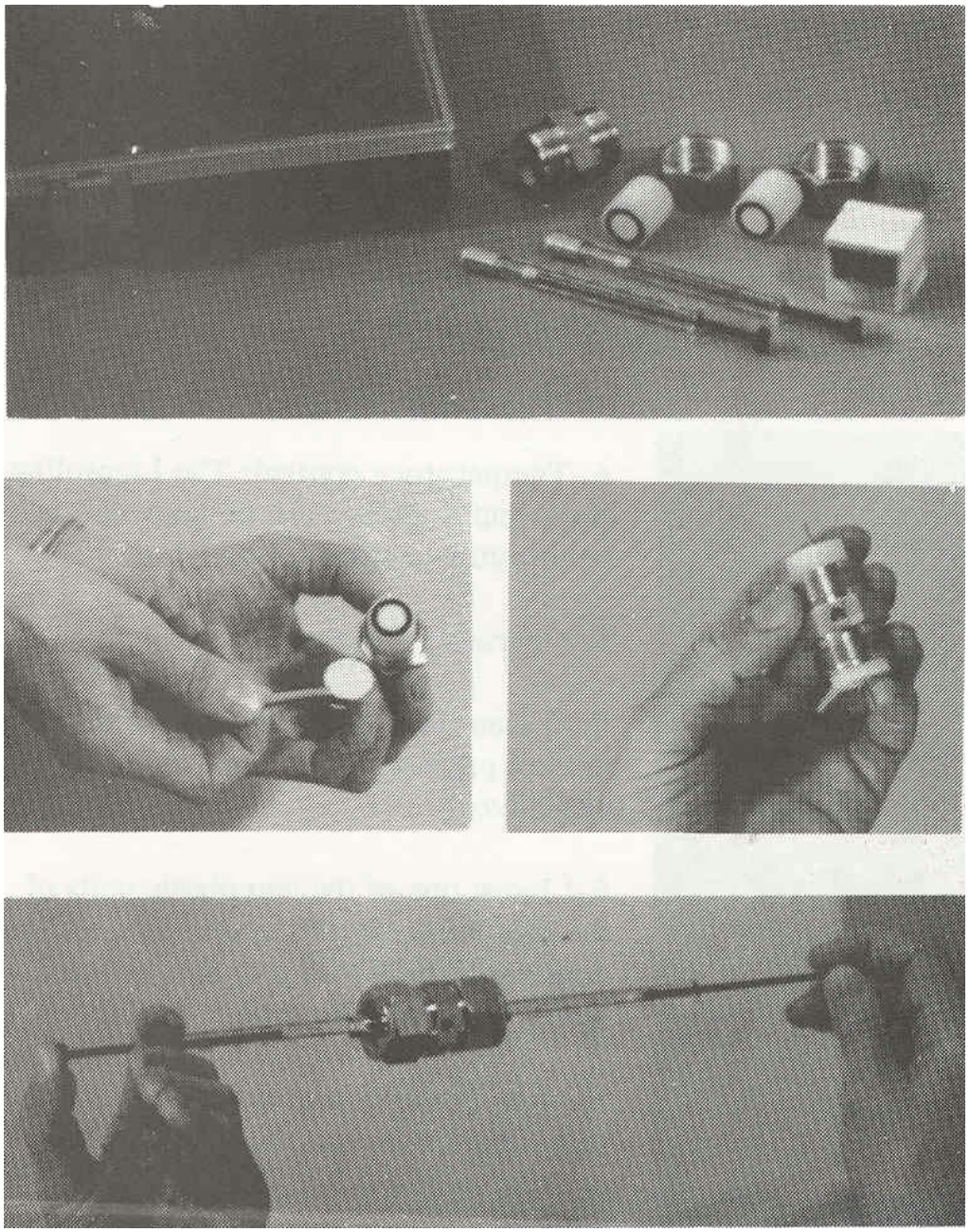

Figure 2.6: Extrusion procedure

grid, mesh 400 (purchased from Plano GmbH, Germany.), previously coated with formvar (polyvinyl formal), support film. The sample was allowed to adsorb to the carbon support film for 10 min. The droplet was carefully removed by touching it with the edge of a blotting paper making sure that the specimen did not completely dry but remained hydrated. Subsequently, 10 μl of the staining solution (phosphotungstic acid 1%, pH 6.8) was pipetted to the carbon support film with the adsorbed specimen. Excess of the staining solution was removed after aproximately 1 min. with the edge of a blotting paper. Specimens were allowed to air-dry. Specimens are stable for at least 2 days and can be kept at room temperature in dark. Carotenoid concentration in liposome suspension is checked spectrophotometrically against control liposmes (without carotenoid load).

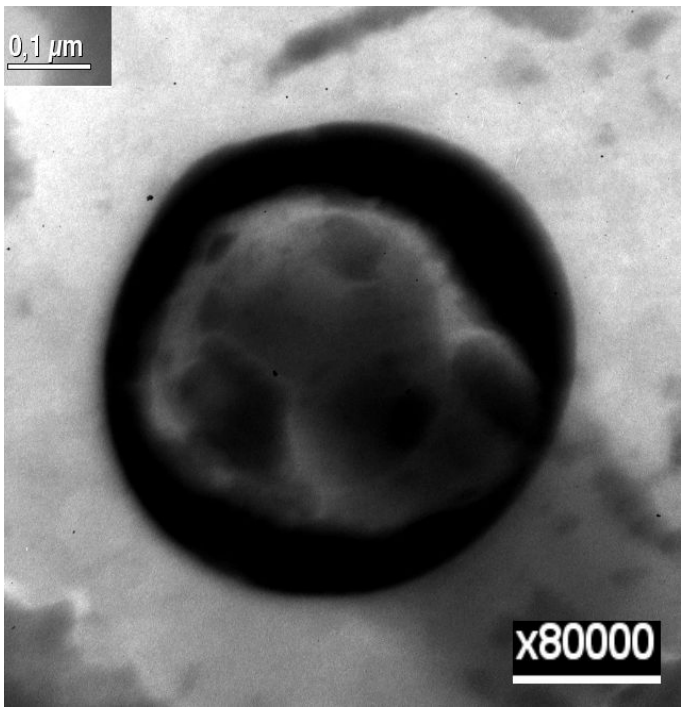

Figure 2.7: Unilamellar liposome. This figure is a negative staining TEM, 80 000 times enlarged photograph of a liposome preparation clearly showing the unilamellar character of the liposome membrane structure. The diameter of the vesicles is about 500 nm.

Conjugated Dienes Measurement To assess lipid peroxidation in lipid bilayer membranes, conjugated dienes are measured. Lipid peroxidation is initiated by challenging unilamellar liposomes with a radical initiator (e.g. AAPH). Using sodium phosphate buffer (100 mM, pH 7.4) unilamellar liposome samples were diluted to a final concentration of 0,1 mg lipid/ml for conjugated diene measurements. 1 ml of unilamellar liposome suspension was pipetted into a quartz cuvette. Lipid peroxidation was initiated by addition of 10 μl of a 50 mM AAPH stock solution to yield a final concentration of 0,5 mM. Conjugated diene formation was measured at 234 nm in a Perkin Elmer spectrophotometer at 37°C.

Lipid Determination The assay determines indirectly the lipid concentration by enzymatic cleavage of the choline moiety from the phospholipid backbone by phospholipase D, hence only choline containing lipids can be determined with this kit. The liberated choline is subsequently oxidized to betaine by choline oxidase with the simultaneous production of hydrogen peroxide. The hydrogen peroxide, which is produced quantitatively, oxidatively couples 4-aminoantipyrine and phenol to yield a color product with a maximum absorption at λ= 505 nm. The kit is provided with buffer, coloring enzymatic reagent and standard. Absorbance of sample is read at 505 nm.

Procedure Three test tubes marked as (*0, ST, X*) containing 1.5 ml of kit's color regent, were mixed with 10 μl of H20, standard (ST) and of sample (X) pipetted into the respective test tubes. Test tubes were vortexed and incubated at 37°C. Absorbance was read at 505 nm against test tube marked *0*.

Final lipid concentration of stock solution was calculated as:

$$\text{Concentration in mg/ml} = (abs.X/abs.ST) * 3$$

Lipid Stock Solution Concentrations of lipid stock solutions dissolved in DCM were determined by pipetting 20 μl of the lipid stock into a glass test tube. DCM was evaporated by warming up the test tube in a hot bath (45-50°C). The dried lipid was suspended in 1 ml of sodium phosphate buffer (100 mM, pH 7.4) by keeping test tube warm and by continuous vortexing. Test tube was marked as lipid stock

suspension (LSS). Three fresh test tubes marked as (*0, ST, LSS*) containing 1.5 ml of kit's color regent, were mixed with 10 μl of H20, standard (ST) and of lipid stock suspension (LSS) pipetted into the respective test tubes. Test tubes were vortexed and incubated at 37°C. Absorbance was read at 505 nm against test tube marked *0*. Final lipid concentratin was calculated as previously described.

1.5 ml of kit's color regent were added to 10 μl of the lipid suspension. Test tubes were vortexed and incubated at 37°C. Absorbance was read at 505 nm against test tube and was left incubating for

2.3.5 TEAC Assay

Materials 2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), ABTS and 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid, Trolox were purchased from Sigma (A-1888 and Cat: 23,881-3 respectively) $K_2S_2O_8$ was obtained from Merck.

Assay The total equivalent antioxidant capacity assay, TEAC assay, described by van den Berg et al.(1999) was used with minor modifications. This assay assesses the total radical scavenging capacity, based on the ability of a compound to scavenge the stable ABTS radical cation ($ABTS^{\bullet+}$).

To form the blue color radical cation $ABTS^{\bullet+}$, 5 ml of a 14 mM ABTS (38.41 mg in 5 ml H_2O) solution was mixed with 5 ml of 4.9 mM $K_2S_2O_8$ (66.22 mg in 50 ml H_2O). The solution was kept in the dark at room temperature and prepared at least 6 hours before use. The solution is stable for at least 2 days. Every prepared solution's radical scavenging activity was calibrated against Trolox. If the radical solution was not totally used during an assay and left for next day, it was recalibrated prior being used again.

The reaction of $ABTS^{\bullet+}$ with antioxidants was determined spectrophotometrically at 734 nm at room temperature. The degree of decolorization is correlated with the antioxidant capacity, which can be converted into corresponding TEAC-values.

Hydrophilic Compounds For the evaluation of hydrophilic antioxidants in an aqueous environment (e.g. blood plasma) the Trolox standard curve was prepared in H_2O. The ABTS$^{\bullet}$ solution was diluted with phosphate buffer saline (PBS), pH 7.4 to a final absorbance of 0.7 ± 0.10.

Lipophilic compounds For lipophilic compounds (e.g. carotenoids or tocopherol), the ABTS$^{\bullet+}$ solution was diluted in ethanol to obtain the same final absorbance as previously indicated. The Trolox standard curve was also prepared in ethanol.

Calculation of TEAC value The TEAC value is the relative antioxidant scavenging capacity of a compound compared to that of Trolox.

To calculate the TEAC value, a Trolox standard curve was prepared with at least four concentration points. The decrease in absorbance of the ABTS$^{\bullet+}$ radical solution caused by each of the Trolox concentrations was registered after 11 min. and the inhibition percentage calculated the following way:

$$Inhib_{ci} = \frac{Abs_{t0} - Abs_{t11}}{Abs_{t0}} x100$$

Where:

$Inhib_{ci}$ is the inhibition caused by a ci Trolox concentration point.

Abs_{t0} is the absorbance of the ABTS$^{\bullet+}$radical solution at time zero.

Abs_{t11} is the absorbance of the ABTS$^{\bullet+}$ radical solution after 11 minutes.

The inhibition is plotted against the respective Trolox concentrations. The calculated linear equation curve out of the Trolox calibration curve, was specific for the ABTS$^{\bullet+}$ radical solution prepared for the day the assay was performed, thus meaning that the ABTS$^{\bullet+}$ radical solution had to be recalibrated if it was used next day.

The TEAC value of an antioxidant compound is therefore determined by calculating it´s inhibition using the calibrated ABTS$^{\bullet+}$ radical solution against Trolox and by correlating it with the Trolox calibration equation curve. The TEAC value

is then expressed in μM Trolox equivalence, that is, the amount μM Trolox needed to obtain the same antioxidant effect than that of the compound investigated.

2.3.6 Statistical Analysis

To evaluate the "5 a Day" intervention study an SPSS data base was created (SPSS for Windows, version 12.0). This data base contained the volunteer's socio-economical data (study begin and end of the study), scores from all seven FFQ, as well as the clinical parameters, biomarker and micronutrient levels' from the blood analysis (T0, T1, T2, T3). Anonymized data from volunteers were assigned in six groups of about 25 individuals and filed according to meeting dates. All analysis were performed with the public domain statistical software R (Development Core Team 2004) version 2.01.

Descriptive Statistics Demographic data and blood parameters were statistically described by frequency tables, means and standard deviations. Descriptive statistics related to the values of vitamins and antioxidants were calculated by using mean and standard deviation at each time point of measurement, namely: base line (T0), one month (T1), two months (T2) and three months (T3).

Analysis of Food Frequency Questionnaires, FFQ The collected food frequency questionnaires were used to verify compliance regarding intake of fruit and vegetables.

For each food item included in the FFQ, intake was assessed in the following frequency categories: never, less than once a week, about once a week, several times a week, about once a day and several times a day.

Compliance was analyzed by establishing two interventional periods. The first period (Pd-1) was defined as: base line to 15-days. The second period (Pd-2) was from 15-days to end of the study.

The nutritional behavior was evaluated using the nutritional index developed by Winkler et al. [58]. The overall Index has a total possible score ranging from 0 to 32. The higher the index score, the better the diet conformed to the dietary guidelines recommendations by the DGE (*Deutsche Gesellschaft für Ernährung*). The

Table 2.7: Two way contingency table for the dynamic migration model showing the three possible nutritional states between two periods.

		to Pd-2		
		A=optimal	B=normal	C=poor
from Pd-1	A=optimal			
	B=normal			
	C=poor			

resulting score was classified according to the three categories defined by Winkler: A=optimal (score grater than 16), B=normal (score between 14 to 16 points) and C=poor nutritional behavior (score less than 13 points). A, B and C represent the current nutritional states of the participants.

Volunteers' compliance was assessed with a dynamic migration model. The frequencies of changes between nutritional states (A, B, C) and intervention periods (Pd-1, Pd-2) were analyzed with a 2 way contingency table (Table 2.7), in order to test: 1) differences between Pd-1 and Pd-2, and 2) the probability to migrate to an upper nutritional state (e.g. from: C to A, or: B to A). The statistical significant level of the model's parameters was taken at 1%.

Longitudinal Analysis For blood parameter data a longitudinal analysis was performed. This analysis was based on a linear growth curve model [63]. This is a two stages hierarchical model. At the first stage individual parameters and intra-individual variability were introduced. At the second stage variability between individuals were defined as random. By denoting y_{it} with a measurement of individual i at time t, this model was written as:

$$y_{i,t} = (\beta_0 + \beta_{0,i}) + (\beta_1 + \beta_{1.i})t + Smoke_i\beta_2 + Gender_i\beta_3 + Age_i\beta_4 + AcuteDisease_i\beta_5 + ChronicDisease_i\beta_6 + \varepsilon_{i,t,}$$

The set of fixed-effects parameters are: the intercept β_0, the slope β_1 of the linear trend. As mentioned above, the parameters used for adjustment were smoke (β_2), gender (β_3), age (β_4), acute disease (β_5), chronic disease (β_6) respectively.

The random effect $\beta_{0,i}$ indicates individual deviation from group mean at the first observation time, $\beta_{1.i}$ individual deviation from the group slope and $\varepsilon_{i,t}$ measurement error.

These three random variables were assumed statistically independent of each other, with mean zero and variances τ_{b0}, τ_{b1} , and τ_{ε} respectively. The parameter of interest in this analysis was the slope of the group β_1 and the individual slopes $\beta_1 + b_{1.I}$. Model parameters were estimated by the restricted maximum likelihood method (REML). The slope of the group is tested with a likelihood ratio test (LRT). Individual slopes are predicted with the best linear unbiased predictors (BLUPs). Asymptotic confidence intervals are reported for all parameters in the model. Model diagnostics and distributional assumptions were assessed by residual analysis. Statistical computations were done with the R language (Development Core Team 2004. version 2.01).

Pair Plot Analysis Pair plot data analysis from Trellis graphics [64] was used to correlate micronutrient levels with one another. A matrix scatter plot was applied. On the diagonal a density lot was use. The upper and lower panels were built with a scatter plot and a simple linear regression to indicate trends between variables.

Correlation Between Food Consumption and Micronutrients For the purpose of analyzing the overall food intake and its influence on the micronutrient and biomarker levels, the FFQ food items were codified to simplify handling of data. Each food item was assigned an "E-number". "E" stands for edible.

A multiple linear regression model was used. The response variable was build as the difference between a measurement at base line and the last observation. This measure of increase was modeled as a function in a set of explanatory variables. These variables were defined as the accumulation of the score intake index for each type of food. The error term of the model was assumed to follow a Gaussian distribution with constant variance. Parameters were estimated by the method of the "Maximum Likelihood". Standard errors were calculated from the inverse of the observed Fisher information matrix. P-values were derived from the Wald statistic.

Bayesian Information Criteria (BIC) The model selection was carried out by the Bayesian Information Criteria (BIC) [65]. This information criterion was adopted to search for a parsimonious model that fit the data [66]. The lower the BIC the better the model explains the data.

A search in the model space was done with a stepwise algorithm. The range of the models examined in the stepwise search was from the simplest model with a constant term, to the most complex model with all variables included. After a potential model was selected, further manually examination of covariates was done.

Chapter 3

Results

3.1 "5 a Day" Intervention Study Results

3.1.1 Evaluation of the Intervention Study Using a Descriptive Statistical Analysis.

The amount of data obtained at the end of the study comprises the biochemical parameters measured in blood (levels of micronutrients, biomarkers of oxidative stress and clinical parameters at four time points T0, T1, T2, T3) and the personal data of the volunteers including, socio-economical background (social situation, work load, education, age, sex, smoking habits, etc.) and the food frequency questionnaires. The following table (3.1) lists the amount of data collected.

The overwhelming amount of data points collected had to be handled in such a way, to reduce the work load of the analysis and yet be efficient enough to obtain clear results from the study.

The first approach for evaluation of the intervention study was to perform a descriptive statistical analysis, giving mean values and standard deviation for each blood parameter obtained at the four time points blood samples were drawn (T0, T1, T2, T3).

Table 3.1: Total amount of data obtained from the "5 a Day" study. Personal data include the socio-economical data and data from the food frequency questionnaires. Biochemical data, include parameters analyzed in the blood samples: micronutrients (e.g. carotenoids and vitamins), biomarkers of oxidative stress (e.g. MDA) and clinical parameters (e.g. total cholesterol, uric acid).

data	variables	time points	subjects	sub-total
socio-economical	68	2	125	59 500
food frequency questionnaire	46	7	125	40 250
biochemical	20	4	125	10 000
			Total	109 750

Analysis of Socio-Economical Data Demographical information about the volunteers such as age, gender, smoking, etc., was included in the evaluation of the data collected.

From the 129 individuals, 112 individuals with complete socio-economical data from all sessions were included for the final descriptive statistical analysis; 16 males and 96 females. Subjects were between 21 and 63 years of age, lowest and highest age, respectively. The average age was 53 $\pm$ 9.9 years. Among the volunteers, 12% were smokers, 35% were ex-smokers, and 53% had never smoked. When asked about to self-estimate their overall health status, only one third of the volunteers considered itself as in good health (33%), 10% considered themself as very healthy and more than the half of the population was either not satisfied with their health or considered it as bad. Self-estimation of overall health status is sumarized in Table 3.2.

When asked about being diseased at the starting of the program, 20% claimed to be acutely diseased, the rest claimed not to have any health problems. From the above mentioned socio-economical parameters smoking, gender, age, acute- and chronic-disease were used further to adjust the analysis (see 3.1.3 on page 76) using a longitudinal statistical analysis for confounders.

Body weight and height to calculate the body mass index, BMI, was measured

Table 3.2: Self-estimated overall health status of 112 individuals completing the intervention study.

Percentages	Self-estimated overall health status
4%	bad
23%	not so good
30%	satisfactory
33%	good
10%	very good

at T0/base line and at the end of the trial (T3). It was expected that due to an increased consumption of fruits and vegetables an overall decrease in the body weight of the participants may be observed. This was, however, not the case. BMI at base line was 26.6 ± 4.4 and 26.0 ± 4.6 at the end of the study (after three months), thus showing no significant difference in weight.

Analysis of Micronutrients, Biomarkers of Oxidative Stress and Clinical Parameters Since previous epidemiological studies have consistently shown that the risk for age-related chronic diseases is correlated with dietary habits, the analysis of micronutrients, biomarkers of oxidative stress and clinical parameters in volunteers' blood throughout the study was of central importance. It was hypothesized that due to the increased consumption of fruits and vegetables, the levels of micronutrients and antioxidants would rise, concomitantly affecting levels of biomarkers of oxidative stress and clinical parameters.

Blood plasma samples of 129 volunteers collected at four time points (sessions), T0 (base line), T1 (1st month), T2 (2nd month) and T3 (3rd month), were analyzed by means of HPLC (see section 2.3.2), for six carotenoids (lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene), two water soluble vitamins (vitamin C and vitamin B6), two lipid soluble vitamins, vitamin E (α-tocopherol) and vitamin A (retinol), and two biomarkers of oxidative stress MDA (as marker for lipidperoxidation) and protein carbonyls (as marker for protein oxidation) the

later being analyzed using ELISA. Homocysteine an independent risk marker for cardiovascular diseases (CDV) was also analyzed by HPLC.

Blood serum was further analyzed for clinical parameters including total cholesterol, HDL- and LDL-cholesterol, triglycerides and uric acid.

The blood parameters were evaluated performing a descriptive statistical analysis as a first approach of evaluation.

The results of the descriptive analysis of blood parameters are summarized in Table 3.3. Shown are plasma levels (mean ± SD) of micronutrients and vitamins, total protein carbonyls, MDA, homocysteine as well as clinical parameters.

Presented are the values measured from participants where a whole set of data [1] at every counseling session (base line (T0), one month (T1), two months (T2) and three months (T3) was available).

[1]The data for the descriptive analysis were taken from volunteers who provided complete data set for one study day.

Table 3.3: Descriptive statistical analysis for blood levels of micronutrients, vitamins, biomarkers of oxidative stress and clinical parameters of individuals during the three months of the intervention study. P-value refers to a significant difference between T3 and T0.

Parameter	mean ± SD			
	base line/T0 (n=112)	T1 (n=114)	T2 (n=113)	T3 (n=108)
Lutein (μmol/L)	0.37 ± 0.16	0.39 ± 0.16	0.40 ± 0.15	0.41 ± 0.16 *
Lycopene (μmol/L)	0.45 ± 0.28	0.50 ± 0.33	0.49 ± 0.30	0.51 ± 0.28 *
α-Carotene (μmol/L)	0.12 ± 0.13	0.19 ± 0.18	0.19 ± 0.17	0.18 ± 0.17 *
β-Carotene (μmol/L)	0.65 ± 0.45	0.87 ± 0.64	0.86 ± 0.65	0.85 ± 0.62 *
β-Cryptoxanthin (μmol/L)	0.35 ± 0.36	0.35 ± 0.28	0.31 ± 0.25	0.27 ± 0.19 *
Zeaxanthin (μmol/L)	0.08 ± 0.07	0.08 ± 0.03	0.08 ± 0.03	0.08 ± 0.03
Vitamin A (Retinol) (μmol/L)	1.35 ± 0.35	1.33 ± 0.35	1.34 ± 0.35	1.33 ± 0.30
Vitamin E (α-Tocopherol) (μmol/L)	28.9 ± 10.3	28.7 ± 11.4	28.4 ± 9.6	29.5 ± 9.80
Vitamin C (μmol/L)	55.1 ± 20.9	57.4 ± 26.9	55.6 ± 23.3	63.6 ± 21.8 *
Vitamin B6 (nmol/L)	48.3 ± 30.9	65.4 ± 39.2	64.0 ± 37.5	64.4 ± 41.6 *
Protein Carbonyls (nmol/mg)	0.79 ± 0.64	0.87 ± 0.9	0.87 ± 0.79	0.80 ± 0.67
MDA (μmol/L)	0.15 ± 0.11	0.17 ± 0.12	0.16 ± 0.11	0.17 ± 0.16
Homocysteine (μmol/L)	11.6 ± 3.6	12.6 ± 4.4	12.2 ± 3.9	11.8 ± 3.5
Uric acid (mg/dL)	3.3 ± 1.0	3.4 ± 0.9	3.9 ± 1.0	3.8 ± 1.0 *
Total cholesterol (mg/dL)	136 ± 37	132 ± 36	162 ± 37	163 ± 33 *
LDL-cholesterol (mg/dL)	73 ± 27	68 ± 26	92 ± 29	91 ± 27 *
HDL-cholesterol (mg/dL)	35 ± 12	33 ± 13	43 ± 14	43 ± 14 *
Triglycerides (mg/dL)	119 ± 86	146 ± 110	132 ± 83	157 ± 99 *

*$p < 0.01$

Table 3.4: P values. Statistically significant differences between base line (T0) and last time point T3 for the two sided paired t-test.

Parameter	p-value	Parameter	p-value
Lutein	< 0.01	Zeaxanthin	0.94
β-Cryptoxanthin	< 0.01	Lycopene	< 0.01
α-Carotene	< 0.01	β-Carotene	< 0.01
Vitamin A (Retinol)	0.47	Vitamin E (α-Tocopherol)	0.55
Vitamin C	< 0.01	Vitamin B6	< 0.01
Protein Carbonyls	0.81	MDA	0.24
Homocysteine	0.93	Uric Acid	< 0.01
Total cholesterol	< 0.01	Triglycerides	< 0.01
LDL-cholesterol	< 0.01	HDL-cholesterol	< 0.01
BMI	0.77		

The significance of the difference of micronutrient levels in vitamins, biomarkers of oxidative stress and clinical parameters was evaluated by a two sided paired t-test ($p< 0.01$). Table 3.4 shows the p-values for the parameters measured. The p-values refer to differences observed between the base line (first time point, T0) and the last time point (T3).

Carotenoids As seen from the previous two tables (Tables 3.3 and 3.4), after three months of the intervention program, from the six carotenoids analyzed, five, lutein, lycopene, α-carotene, β-carotene and β-cryptoxanthin showed a significant difference ($p < 0.01$) in their plasma levels comparing T0/base line and T3. In contrast to lutein, lycopene, α-carotene and β-carotene, which significantly increased, β-cryptoxanthin levels significantly decreased. For zeaxanthin no significant change over time was observed, levels remained at around 0.08 μmol/L. The time course for each blood parameter are given the following figures.

Lutein Starting at 0.37 μmol/L at T0, levels of lutein continuously increased in an almost linear manner reaching its highest value of 0.41 μmol/L at T3. Figure 3.1 shows means and 95% confidence intervals for each time point of measurement.

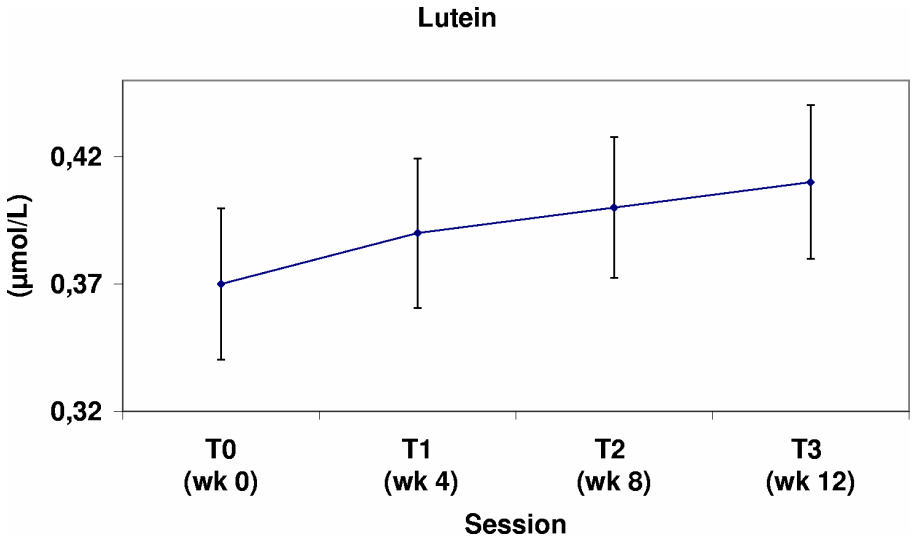

Figure 3.1: Lutein plasma levels, means and 95% confidence intervals for each time point.

Lycopene Lycopene blood plasma values increased during the study (Fig.3.2). Between T1 and T2 a slight decrease is observed. The maximum value is observed at around 0.51 μmol/L at T3.

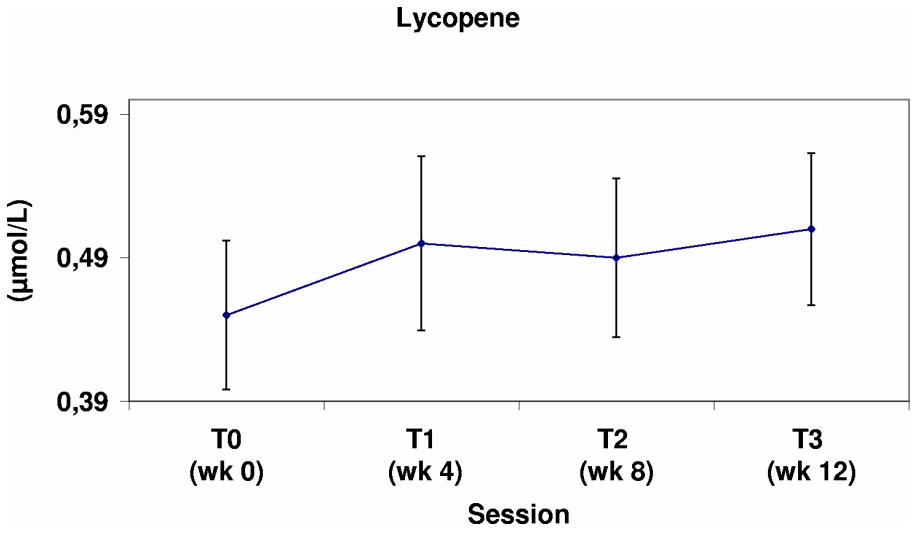

Figure 3.2: Lycopene plasma levels, means and 95% confidence intervals for each time point.

α-Carotene α-Carotene (Fig.3.3) blood plasma levels increase by almost 60% within the first month of intervention (from 0.12 $\pm$ 0.13 μmol/L to 0.19 $\pm$ 0.18 μmol/L). The highest value was reached at T1 (0.19 μmol/L). At T3 a slight decrease is observed.

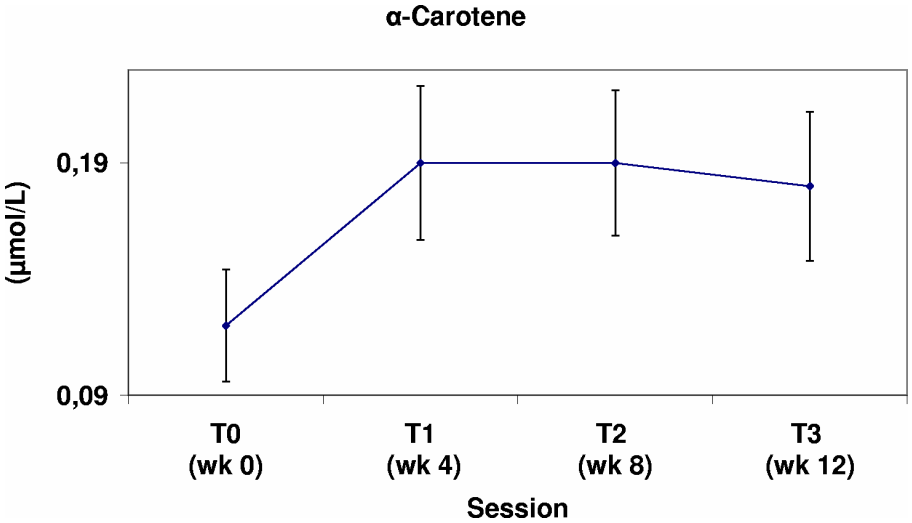

Figure 3.3: α-Carotene plasma levels, means and 95% confidence intervals for each time point.

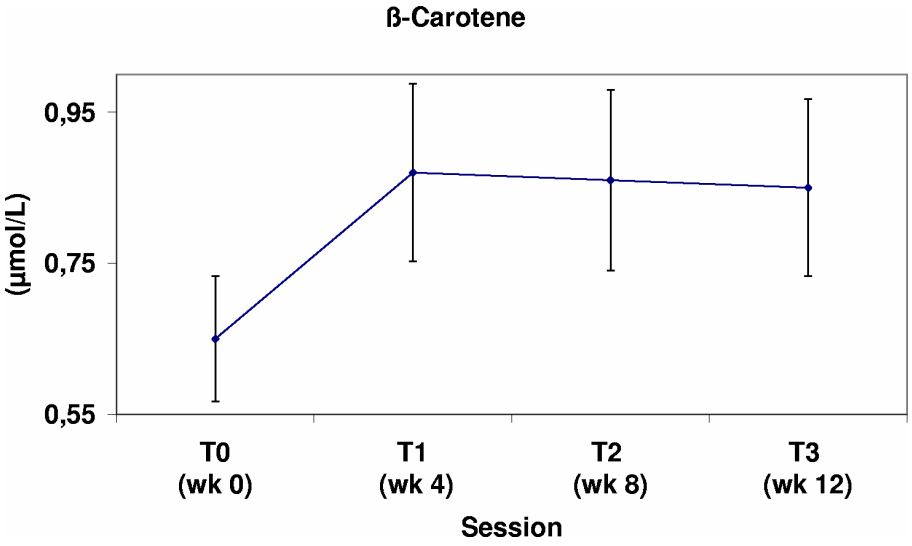

Figure 3.4: β-Carotene plasma levels, means and 95% confidence intervals for each time point.

β-Carotene β-Carotene blood plasma levels show a similar curve as α-carotene (Fig.3.4). Levels increased by almost 35% within the first month of intervention (from 0.65 $\pm$ 0.45 μmol/L to 0.87 $\pm$ 0.64 μmol/L). The highest value was detected at T1.

β-Cryptoxanthin β-Cryptoxanthin is the only carotenoid in blood plasma showing a significant decrease. The lowest level of 0.27 $\pm$ 0.19 μmol/L was observed at T3. Figure 3.5 shows that levels of this carotenoid decrease from T1 almost linearly.

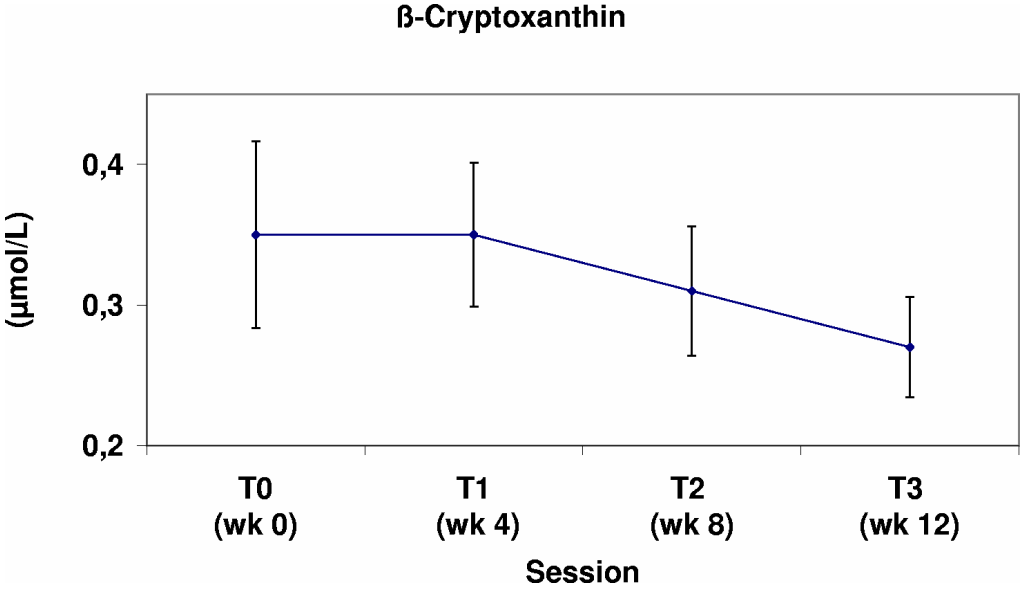

Figure 3.5: β-Cryptoxanthin plasma levels, means and 95% confidence intervals for each time point.

Zeaxanthin Fig. 3.6 shows that plasma levels of zexanthin were not affected by the intervention. The base line value of 0.08 μmol/L did hardly change.

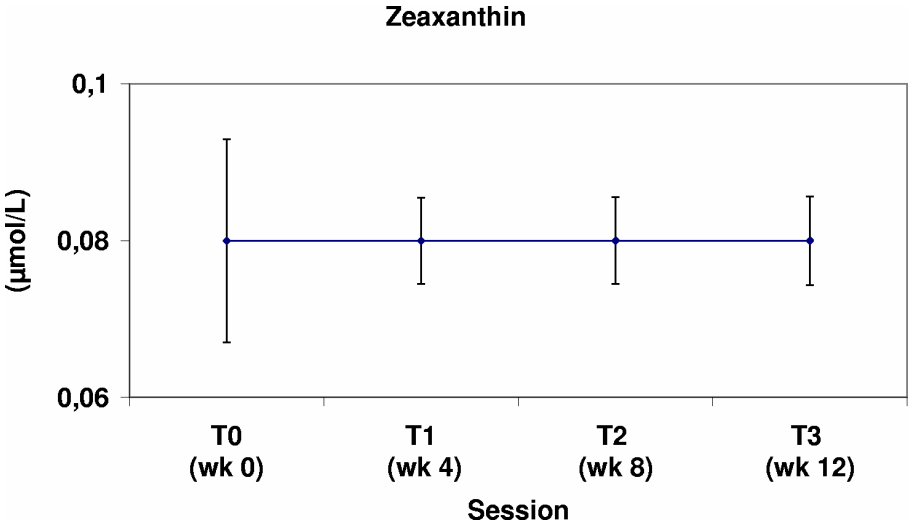

Figure 3.6: Zeaxanthin plasma levels, means and 95% confidence intervals for each time point.

Vitamins Among the four vitamins analyzed, the levels of lipid soluble vitamins, vitamin A (retinol) and vitamin E (α-tocopherol) did not show any significant change. In contrast, the water soluble vitamins, C and B6 were significantly higher at the end of the intervention (12 weeks, T3).

Lipid Soluble Vitamins The following diagrams show levels of vitamin A (retinol) and vitamin E (α-tocopherol) at the four time points of measurement (Figures 3.7 and 3.8, respectively).

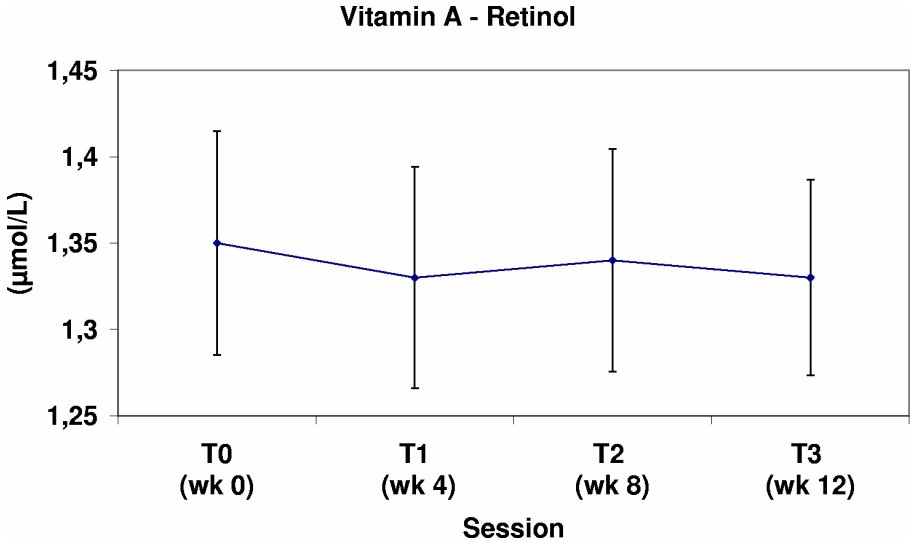

Figure 3.7: Blood levels of vitamin A, means and 95% confidence intervals for each time point.

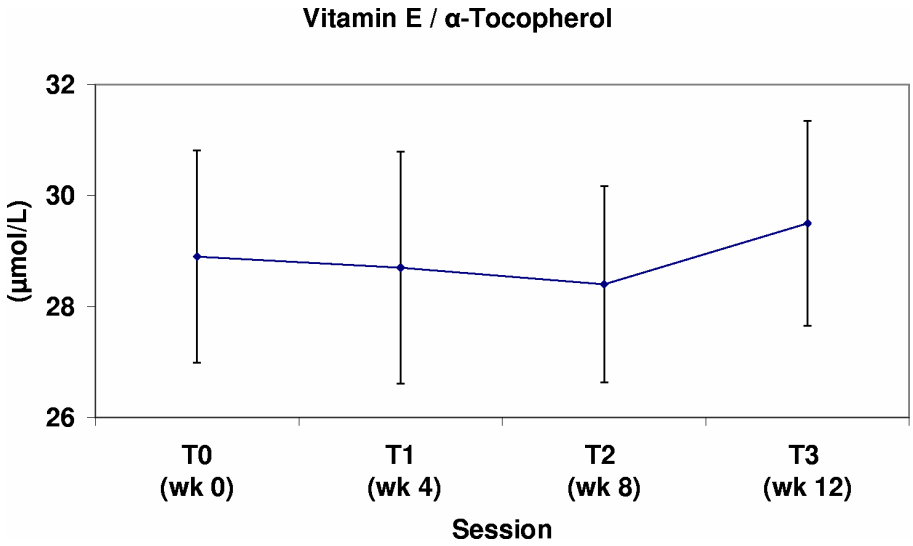

Figure 3.8: Blood levels of vitamin E, means and 95% confidence intervals for each time point.

Water Soluble Vitamins Figure 3.9 shows blood levels of vitamin C and vitamin B6 at the four time points of measurements. Highest value for vitamin C was observed at T3 (63.4 ± 41.6 μmol/L) which is significantly different from the base line value (55.1 ± 20.9 μmol/L). Blood levels of vitamin B6 increase up to 35% within the first month of intervention. At T3 blood levels of vitamin B6 are highest (around 64 nmol/L) and significantly different from T0.

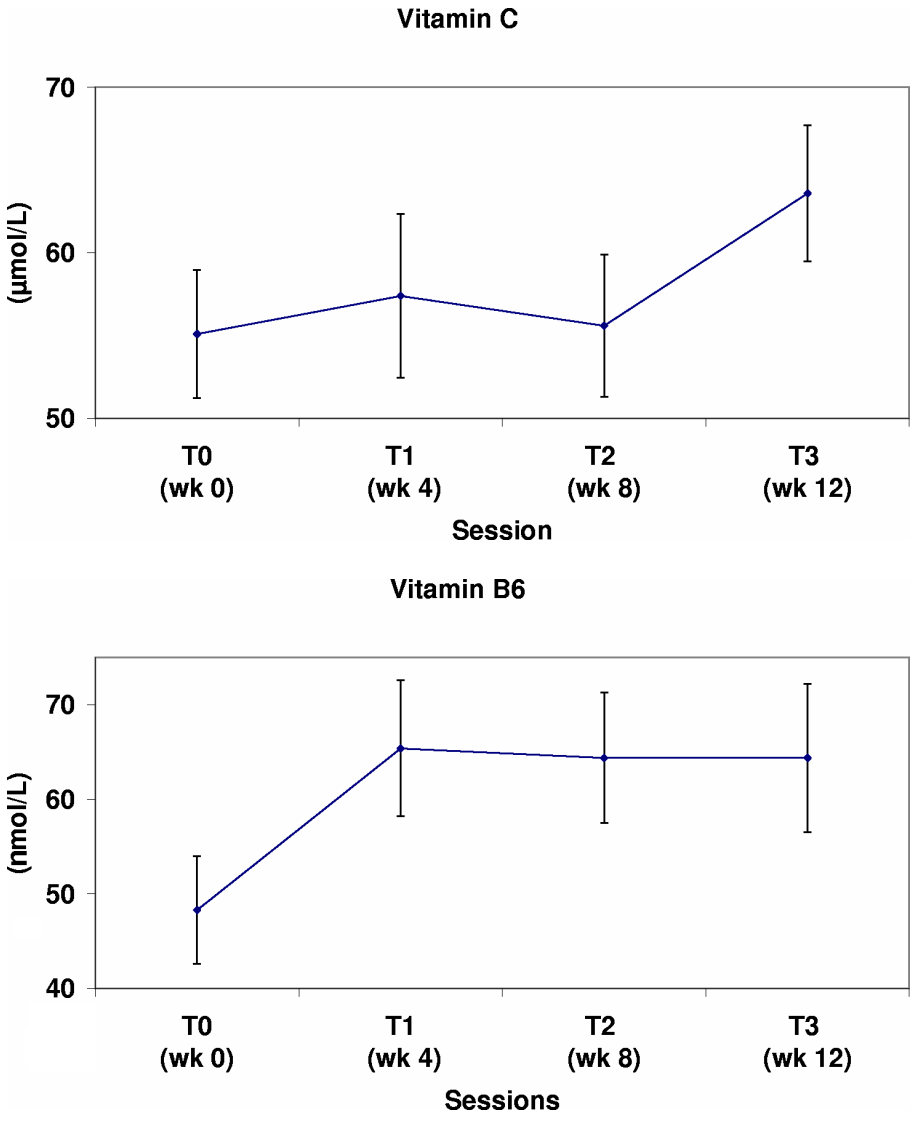

Figure 3.9: Blood levels of vitamin C and vitamin B6, means and 95% confidence intervals for each time point.

Biomarkers of Oxidative Stress The levels of biomarkers of oxidative stress, MDA and total protein carbonyls, did not show any significant change comparing base line and T3. Total plasma homocysteine, was not affected by the dietary intervention.

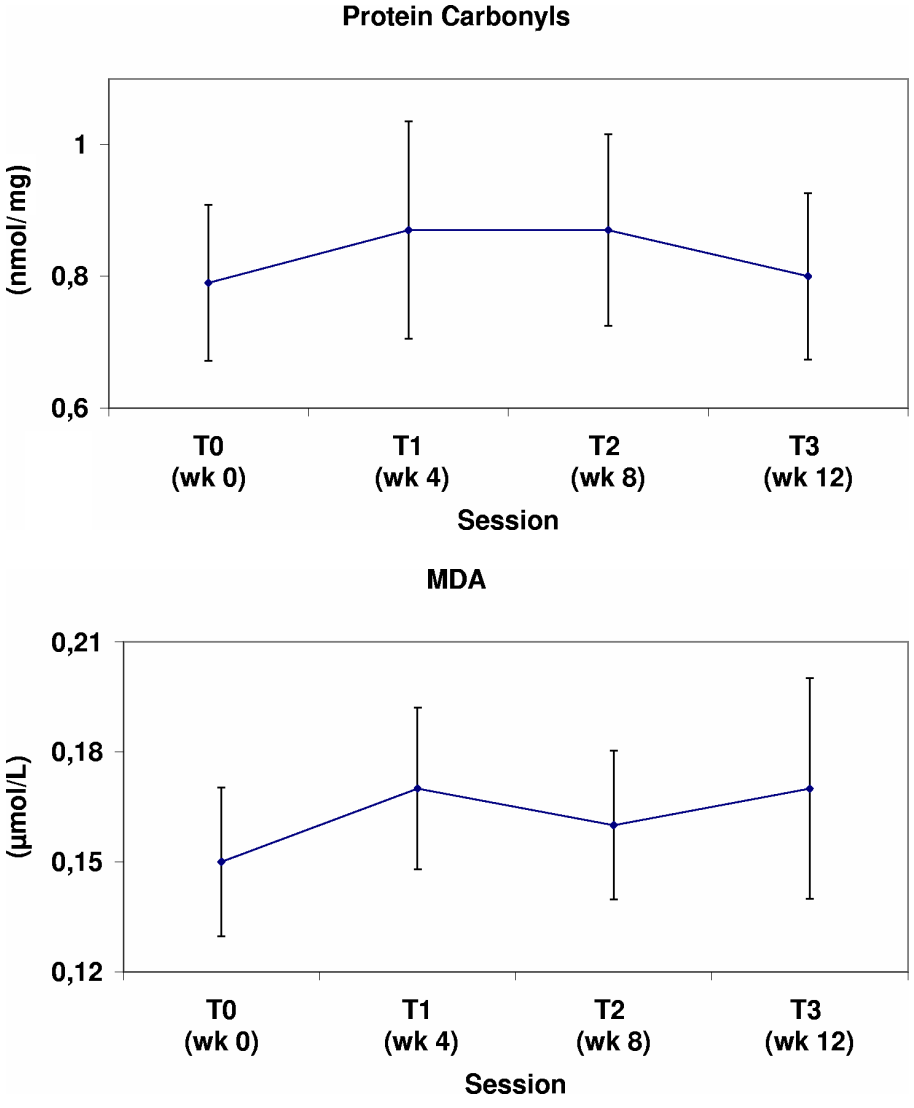

Figure 3.11: Blood levels of biomarkers of oxidative stress, means and 95% confidence intervals for each time point.

Levels of homocysteine remained below 15 μmol/L. Although commonly listed within the clinical parameters, uric acid can be regarded as a metabolic marker for cardiovascular diseases. High levels of this metabolic product have been positively correlated to cardiovascular mortality. Levels of uric acid where significantly higher after the intervention study (3.8 $\pm$ 1.0) but stayed below the risk levels of 7.0 mg/dL and 5.7 mg/dL for males and females, respectively. Figure 3.12 shows levels of homocysteine and uric acid at each time point of measurement.

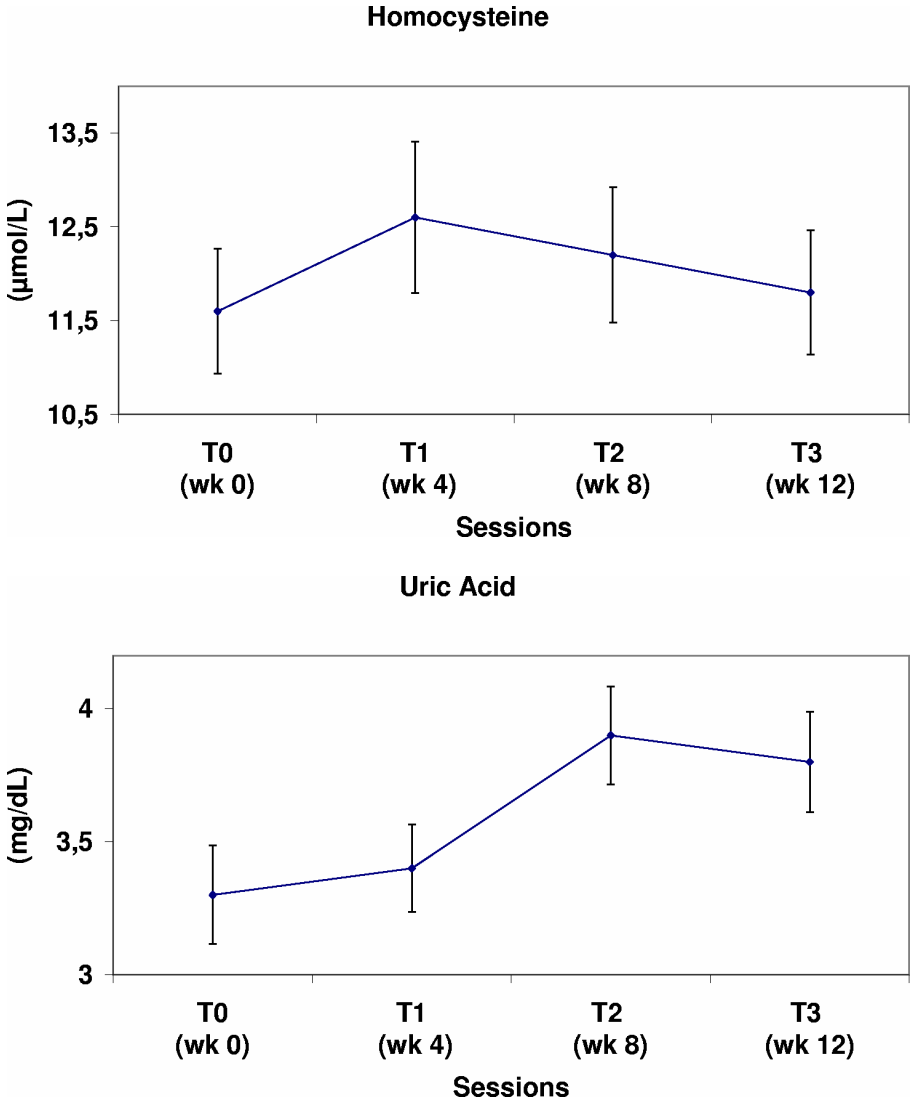

Figure 3.12: Blood levels of biomarkers of oxidative stress, means and 95% confidence intervals for each time point.

Clinical Parameters A statistically significant increase was determined for the clinical parameters, cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides (Figs. 3.13 and 3.14).

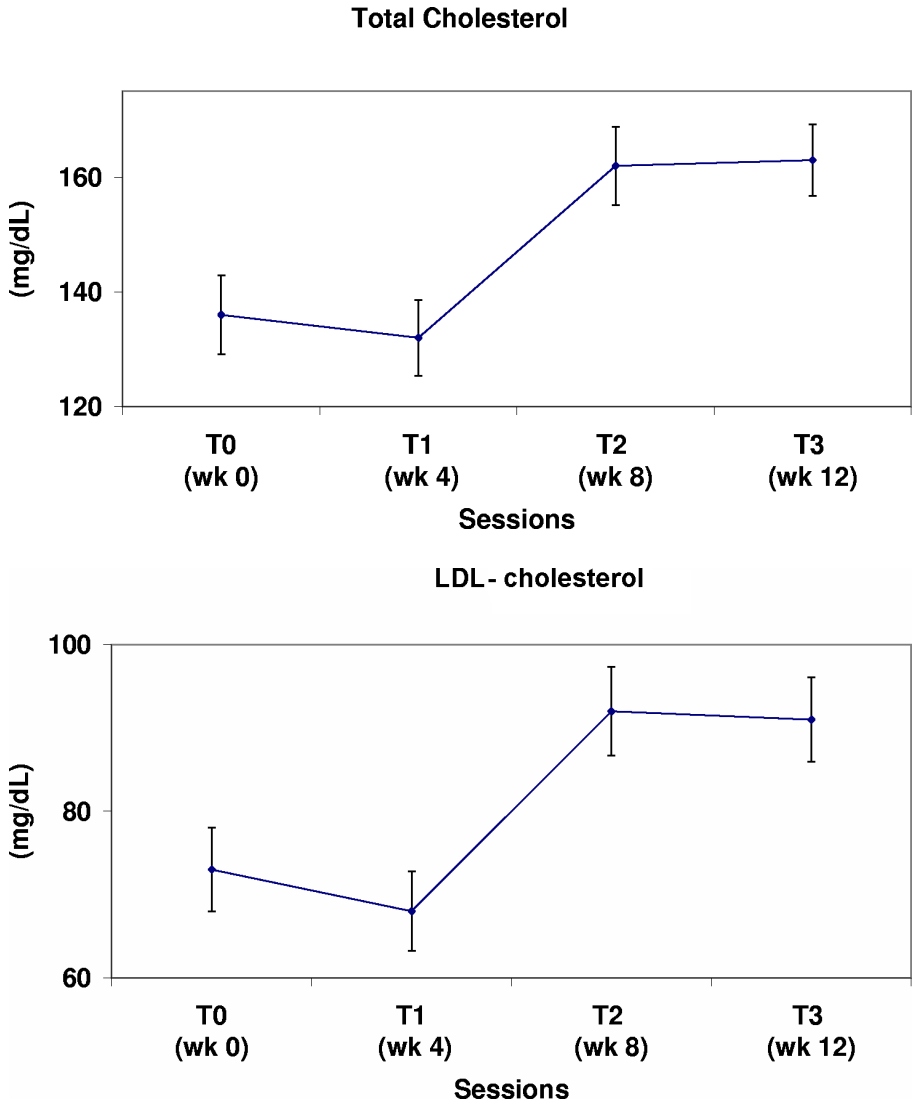

Figure 3.13: Blood levels of clinical parameters, means and 95% confidence intervals for each time point.

The higher levels observed at the end of the study for total cholesterol, LDL-cholesterol and HDL-cholesterol were still within the normal range (see Table 3.6). HDL-cholesterol, was closer to the desired range of > 55 mg/dL and > 65 mg/dL

for males and females, respectively. An interesting observation is the similarity in the shape of the curves of total cholesterol, LDL- and HDL-cholesterol. All curves show a decrease at T1. At T2, levels of this three parameters are significantly higher than T0 or T1, and remain so also at T3. Blood levels of triglycerides were significantly higher at T3 compared to base line values. A decrease in triglycerides levels is observed between T1 and T2.

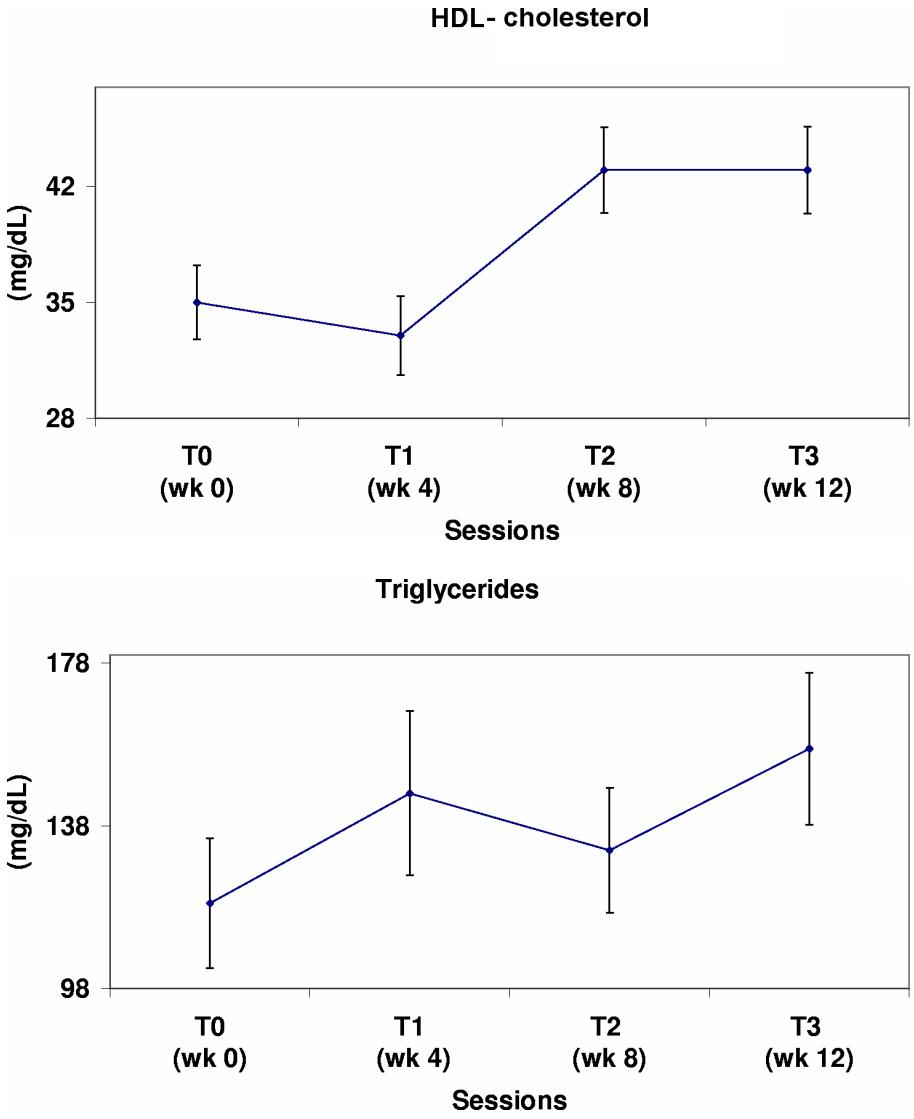

Figure 3.14: Blood levels of clinical parameters, means and 95% confidence intervals for each time point.

Range Despite the fact that it was possible to detect statistical differences between base line values and T3, large standard deviations were observed, indicating large variations of values among individuals. Table 3.5 gives an overview of the ranges measured for each parameter. Although this values (minimum and maximum observed) are not representative for the entire population, they give an idea of the large variation of values observed within the study population (Table 3.3).

Carotenoids Range For most carotenoids, range was generally constant in maximum and minimum values at all time points. For example, lutein minimum and maximum values prevail constant throughout the intervention trial: about four times lower and two and half times higher than the mean (Table 3.5).

In the case of lycopene, maximum level was 1.9 μmol/L. This level is about four times higher compared to the mean of 0.50 μmol/L. The minimum value of 0.06 μmol/L was observed at base line.

The carotenoid showing the lowest levels was α-carotene. The minimum value was observed at 5 nmol/L, the maximum 0.98 μmol/L.

Extreme values were found for β-carotene, where the minimum is sixty five times lower the mean and two hundred seventy times lower than the maximum.

An interesting finding corresponding to the observations made in the descriptive analysis is that for β-cryptoxanthin the observed maximum and minimum decreases also. The maximum observed at T0 is almost eight-fold the mean (2.9 vrs. 0.35 μmol/L). At the end of the study the maximum observed (as well as the minimum) falls to half of the initial value (1.4 μmol/L and 0.03 μmol/L respectively), showing thus a negative trend as observed in the mean.

Zeaxanthin range prevails constant until the end of the study where the minimum (around 0.03 μmol/L) remains about three times lower the mean and the maximum (between 0.3 μmol/L and 0.5 μmol/L) a six fold above it.

Vitamin Ranges Ranges for the lipid soluble vitamins, vitamin A (retinol) and vitamin E (α-tocopherol) remained constant throughout the study. Only vitamin

Table 3.5: Range. Minimum and maximum values observed at different time points

	T0		T1		T2		T3	
Parameter	min	max	min	max	min	max	min	max
Lutein (μmol/L)	0.10	0.98	0.12	1.1	0.11	0.92	0.11	0.99
Lycopene (μmol/L)	0.06	1.4	0.06	1.9	0.08	1.6	0.08	1.5
α-Carotene (μmol/L)	0.005	0.91	0.014	0.97	0.010	0.97	0.014	0.98
β-Carotene (μmol/L)	0.01	2.7	0.03	2.9	0.03	3.5	0.04	3.2
β-Cryptoxanthin (μmol/L)	0.06	2.9	0.03	1.8	0.03	1.8	0.03	1.4
Zeaxanthin (μmol/L)	0.03	0.54	0.03	0.41	0.03	0.49	0.02	0.36
Retinol (μmol/L)	0.68	2.38	0.67	3.03	0.66	2.40	0.65	2.59
α-Tocopherol (μmol/L)	13.3	61.2	5.9	87.7	5.4	66.4	10.3	87.0
Vitamin C (μmol/L)	9.0	127.9	1.9	157.3	1.7	121.3	14.9	140.5
Vitamin B6 (nmol/L)	9.2	170.3	13.7	245.4	13.3	606.0	10.7	238.0
Protein Carbonyls (nmol/mg)	13.0	260.4	12.7	537.9	8.02	461.6	12.4	435.1
MDA (μmol/L)	0.05	0.82	0.04	0.84	0.06	0.87	0.06	1.66
Homocysteine (μmol/L)	4.7	25.2	5.9	27.6	4.8	24.9	5.9	27.7
Uric acid (mg/dL)	1.6	7.0	1.6	6.8	2.1	7.6	1.8	7.1
Total cholesterol (mg/dL)	63	278	51	228	92	285	94	271
LDL-cholesterol (mg/dL)	25	208	25	177	45	213	42	197
HDL-cholesterol (mg/dL)	15	69	11	69	19	86	19	103
Triglycerides (mg/dL)	28	605	33	806	39	620	33	548

E showed some variations in the minimum values.

Water soluble vitamins showed high variation in maximum and minimum values. Vitamin C at T1 had a maximum of 157.3 μmol/L. At the same time point the lowest value observed was of 1.9 μmol/L.

Highest observed vitamin B6 value was at T2 (606 nmol/L) almost ten fold the mean value (64 nmol/L) at this time point.

Biomarkers of Oxidative Stress Range Among the markers for oxidative stress, major variations between maximum and minimum were observed for protein carbonyls at all time points. At T1 the highest value observed was of 537.9 nmol/mg, which is over 600 times higher than the mean (0.87$\pm$ 0.9 nmol/mg).

MDA did not show such high variations between maximum and minimum. Maximum values were about four times higher than the mean, minimum values were only a third of the mean.

Homocysteine variation was quite low.

Clinical Parameters Range In the group of the clinical parameters, uric acid levels did not show extreme values. The highest value of 7.6 mg/dL observed at T2 is close to the recommended value of 7.0 mg/dL. The minimum observed was of 1.6 mg/dL at T0 and T1. Levels beyond the recommended values for total cholesterol were also detected. At T2 a maximum of 285 mg/dL was observed, similar maximum values were observed also at the other time points. LDL-cholesterol levels did not show extreme values. Also no extreme values were observed for HDL-cholesterol levels. Triglycerides levels, showed some extreme values. A peak value of 806 mg/dL was observed at T1, which is a 5.5 fold of the mean of 146 mg/dL at this point. Such a high value of triglycerides is four times above the recommended value of $<$ 200 mg/dL.

The extreme values observed in the study are reflected in the high standard deviations observed, and indicate individual risks of some volunteers. It also points out that several individuals had already high levels in micronutrients, biomarkers of oxidative stress, clinical parameters prior starting intervention with the "5 a day" controlled diet.

Table 3.6: Highest and lowest values observed throughout the study. They are compared with the values observed at the end of the trial (T3). The gender dependent recommended values are listed on the last column.

		Values observed		T3	Recommended*	
Parameter	unit	max.	min.	mean ±s.d.	male	female
Lutein	(μmol/L)	0.99	0.10	0.41 ± 0.16	> 0.25*	
Lycopene	(μmol/L)	1.9	0.06	0.51 ± 0.28	> 0.40*	
α-Carotene	(μmol/L)	0.98	0.005	0.18 ± 0.17	> 0.05*	
β-Carotene	(μmol/L)	3.5	0.01	0.85 ± 0.62	> 0.40*	
β-Cryptoxanthin	(μmol/L)	2.9	0.03	0.27 ± 0.19	> 0.20*	
Zeaxanthin	(μmol/L)	0.54	0.02	0.08 ± 0.03	> 0.05*	
Retinol	(μmol/L)	3.03	0.65	1.33 ± 0.30	> 1.15	
α-Tocopherol	(μmol/L)	87.7	10.3	29.5 ± 9.80	> 20	
Vitamin C	(μmol/L)	157.3	1.7	63.6 ± 21.8	> 30	
Vitamin B6	(nmol/L)	606	9.2	64.4 ± 41.6		
Protein Carbonyls	(nmol/mg)	537.9	8.02	0.80 ± 0.67		
MDA	(μmol/L)	1.66	0.04	0.17 ± 0.16		
Homocysteine	(μmol/L)	27.7	4.7	11.8 ± 3.5	< 15	
Uric acid	(mg/dL)	7.6	1.6	3.8 ± 1.0	< 7.0	< 5.7
Total cholesterol	(mg/dL)	285	51	163 ± 33	< 200	
LDL	(mg/dL)	213	25	91 ± 27	< 160	
HDL	(mg/dL)	103	11	43 ± 14	> 55	> 65
Triglycerides	(mg/dL)	806	33	157 ± 99	< 200	

* = There are no recommended values for carotenoids.

Table 3.6 also presents estimated optimal values[2] for the parameters measured. Included are means and standard deviations at T3 was well as the highest and lowest observed values from all time points. No optimal values have yet been established for protein carbonyls and MDA.

[2]There are no recommended values for carotenoids. The values presented in Table 3.6 are suggested target values based on existing literature.

3.1.2 Summary of the Evaluation of the Intervention Study Using a Descriptive Statistical Analysis

From Table 3.6 it can be seen that at the end of the intervention study all mean values of micronutrient levels in blood plasma are shifted above the desired recommended values. The large standard deviations observed for some of the parameters analyzed can be explained by the extreme ranges observed for minimum and maximum values due to interindividual variation. Worth mentioning are those extreme values which are an order of magnitude higher (zeaxanthin, β-cryptoxanthin and α-carotene) or lower (α-carotene and β-carotene) than the recommended. For biomarkers of oxidative stress no recommended values have yet been established. Although significant higher levels of antioxidants were observed, levels of biomarkers of oxidative stress were not influenced by the three month intervention. All means of clinical parameters were also found to be within the normal range at the end of the study.

3.1.3 Evaluation of the Intervention Study Using a Longitudinal Analysis. Time Dependent Linear Growth Statistical Model.

A shortcome of the descriptive statistical analysis is that it only gives a general overview of a parameter by means and standard deviations at a certain time point. Hence, this first approach to evaluate results of the intervention study does not discriminate how parameters (micronutrients and biomarkers of oxidative stress) change time dependently from T0 to T3.

In order to have a deeper insight into the whole study results, a longitudinal analysis was introduced which is based on a linear growth statistical model adjusted for the confounders smoking, gender, age, acute disease and chronic disease (for details see 2.3.6). This model describes the data with two parameters: β_0 and β_1. β_0 is the corrected mean value of base line and has μmol/L, nmol/L, etc. as unit.

β_1 is the rate of increase/decrease of the corrected blood levels during the intervention period and has μmol/L month, nmol/L month, etc. as a unit.

Starting at β_0, the parameter of interest rises (or decreases) linearly at a constant rate($\beta 1$) assuming the study population would continue with the same diet pattern they adhered to during the study.

Table 3.7, summarizes the results obtained with the linear growth statistical model for the general trend of the 112 individuals. Significances indicated in the Table show that there was statistical significant change regarding the rate of increase ($\beta 1$) in the levels of the micronutrients lutein, β-cryptoxanthin, lycopene, α and β-carotene, vitamin C and vitamin B6. Also the clinical parameters uric acid, total cholesterol, LDL-cholesterol, HDL-cholesterol and tryglicerides showed a statistically significant change. The data presented in the table have been adjusted for confounders. Thus, values are different from the ones obtained for the same parameter in the descriptive statistical analysis.

Table 3.7: Longitudinal analysis. β_0 and β_1 obtained from the linear growth model adjusted for smoking, gender, age, acute-disease and chronic-disease.

Parameter	units	T0 (β_0)	Rate of Increase (β_1)	units
Lutein	μmol/L	0.11 ± 0.10	0.01 ± 0.0036**	μmol/L/month
Lycopene	μmol/L	0.39 ± 0.18	0.012 ± 0.008**	μmol/L/month
α-Carotene	μmol/L	0.06 ± 0.09	0.014 ± 0.004***	μmol/L/month
β-Carotene	μmol/L	0.12 ± 0.34	0.054 ± 0.01***	μmol/L/month
β-Cryptoxanthin	μmol/L	0.28 ± 0.13	-0.031 ± 0.007**	μmol/L/month
Zeaxanthin	μmol/L	0.04 ± 0.02	0.001 ± 0.002	μmol/L/month
Vitamin A (Retinol)	μmol/L	1.45 ± 0.19	-0.003 ± 0.006	μmol/L/month
Vitamin E (α-Tocopherol)	μmol/L	15.9 ± 5.9	0.08 ± 0.21	μmol/L/month
Vitamin C	μmol/L	24.9 ± 11.4	2.3 ± 0.79**	μmol/L/month
Vitamin B6	nmol/L	53.4 ± 19.8	4.3 ± 1.19**	nmol/L/month
Protein Carbonyls	nmol/mg	113.2 ± 42.9	0.8 ± 2.1	nmol/mg/month
MDA	μmol/L	0.26 ± 0.07	0.004 ± 0.005	μmol/L/month
Homocysteine	μmol/L	25.6 ± 16.24	3.21 ± 3.23	μmol/L/month
Uric acid	mg/dL	4.04 ± 0.51	0.218 ± 0.02***	mg/dL/month
Total cholesterol	mg/dL	77.7 ± 19.3	10.35 ± 1.09***	mg/dL/month
LDL-cholesterol	mg/dL	39.4 ± 15.2	6.58 ± 0.79***	mg/dL/month
HDL-cholesterol	mg/dL	14.8 ± 7.5	3.7 ± 0.4***	mg/dL/month
Triglycerides	mg/dL	141.5 ± 51.6	10.7 ± 2.69***	mg/dL/month

**p < 0.01; ***p < 0.001

Lutein Lutein values for β_1 indicate a significant increase of 0.01 $\pm$ 0.0036 μmol/L/month starting at β_0 of 0.11 $\pm$ 0.10 μmol/L.

Lycopene Lycopene levels in the study population rise at a rate of 0.012 $\pm$ 0.008 μmol/L per month. The rate of increase of lycopene in comparable to that of lutein. β_0 of lycopene is about four times higher, than the β_0 of lutein.

α-Carotene The rate of increase for α-carotene was a significant at $p < 0.001$. The value of β_1(0.014 μmol/L/month) of this carotenoid was in a similar range as for lutein and lycopene. Base line value of β_0, 0.06 μmol/L, was about half of the value for lutein and about six times lower than lycopene.

β-Carotene β-carotene has the highest rate of increase of all carotenoids (0.054 $\pm$ 0.01 μmol/L) with a significance of $p < 0.001$. This is five times higher than lutein although the β_0 value are comparable (0.12 and 0.11 μmol/L).

β-Cryptoxanthin The statistically significant lower levels of β-Cryptoxanhtin, previously observed in the descriptive statistical analysis (T0 vs. T3), were confirmed in the linear growth model. The β_1 value is negative, indicating a decrease. The decrease was statistically significant ($p < 0.01$) and corresponds to a loss of 0.031 μmol/L/month.

Zeaxanthin The rate of increase of zeaxanthin was not significant, confirming the observations made in the descriptive statistical analysis were zeaxanthin blood levels did not change during the intervention study.

Vitamin A, Retinol The rate of increase (β_1) of vitamin A (retinol) was not significant (0.003 $\pm$ 0.006 μmol/L/month), confirming the observations made in the descriptive statistical analysis, where this lipid soluble vitamin did not show significant changes comparing T0 and T3. The base line value of vitamin A was around 1.45 μmol/L.

Vitamin E, α-Tocopherol The rate of increase (β_1) of vitamin E (α-tocopherol) was not significant confirming also the observations made in the descriptive statistical analysis. The base line value of vitamin E was the highest of all analyzed lipid soluble micronutrients, 15.9 $\pm$ 5.9 μmol/L.

Vitamin C The rate of increase (β_1) of vitamin C was statistically significant, with an increase of 2.3 $\pm$ 0.79 μmol/L/month, confirming the results of the descriptive statistical analysis. The base line value (β_0) of vitamin C was 24.9 μmol/L.

Vitamin B6 The rate of increase (β_1) of vitamin B6 was significant, with an increase of 4.3 $\pm$ 1.19 nmol/L/month, confirming the results of the descriptive statistical analysis. The base line value (β_0) of vitamin B6 was around 53.4 nmol/L.

Protein Carbonyls No statistical significant change was found for β_1.

MDA No statistical significant change was found for β_1.

Homocysteine No statistical significant change was found for β_1.

Uric Acid The rate of increase (β_1) of uric acid was statistically significant, with a monthly increase of 0.22 $\pm$ 0.02 mg/dL, confirming the observations made in the descriptive statistical analysis. The base line value (β_0) of around 4.04 mg/dL is in the same range as that observed in the descriptive statistical analysis (3.3 $\pm$ 1.0 mg/dL).

Total Cholesterol Total cholesterol showed a significant rate of increase (β_1) of 10.35 $\pm$ 1.09 mg/dL/month. The base line value (β_1) of total cholesterol was observed to be approximately 78 mg/dL.

LDL-Cholesterol LDL-cholesterol showed a significant rate of increase (β_1) of 6.58 $\pm$ 0.79 mg/dL/month. The base line value (β_0) of LDL-cholesterol was observed at approximately 39 mg/dL about half the value compared to the base line value (β_0) of total cholesterol 78 mg/dL.

HDL-Cholesterol HDL-cholesterol had a sttistically significant rate of increase (β_1) of 3.7 $\pm$ 0.4 mg/dL/month. HDL-cholesterol showed the lowest $\beta 0$ and $\beta 1$ compared to total-cholesterol and LDL-cholesterol.

Triglycerides Triglycerides was the only clinical parameter, which after adjustments for the linear growth statistical model, had a higher base line value (β_0) than that observed for the (non-adjusted) descriptive statistical analysis (141.5 $\pm$ 51.6 mg/dL and 119 $\pm$ 86 mg/dL, respectively). The rate of increase (β_1), 10.7 $\pm$ 2.69 mg/dL/month was statistically significant.

3.1.4 Summary of the Time Dependent Linear Growth Statistical Model, Longitudinal Analysis

As it can be taken from table 3.7, the rate of increase β_1 was statistically significant for lutein, lycopene, α-carotene ($p < 0.01$) and β-carotene ($p < 0.001$), as well as for the water soluble vitamins vitamin B6, vitamin C ($p < 0.01$).

Concerning β-cryptoxanthin a significantly negative trend was found ($p < 0.01$), which confirmed the negative trend determined in the descriptive analysis.

Other carotenoids, zeaxanthin and lipid soluble vitamins A (retinal) and vitamin E (α-tocopherol), showed no change in the rate of increase when analyzed with the linear growth model. Short-term intervention for 3 months did not significantly affect plasma levels of MDA and protein carbonyls. The clinical parameters uric acid, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides showed a positive statistically significant rate of increase.

3.1.5 Trends of β_1 for Individual Subjects, Histograms

The results evaluated with the linear growth statistical model, are described with two parameters: a base line value (β_0) and a rate of increase (β_1) for an investigated parameter. Interesting differences regarding the responses to the intervention were obtained when each individual was observed separately. The distribution of individual β_1, of an investigated parameter, can be graphically shown in a histogram.

The values for the individual rate of increase, β_1, are given in: parameter´s concentration in the blood/ month (e.g. μmol/L/month).

The bars of the histogram which are centered around zero (0.0), correspond to the group of subjects with no positive or negative shift in the *parameter´s trend*; bars left shifted (< 0.0) or right shifted (> 0.0) indicate a decrease (-β_1) or increase ($+\beta_1$). The significances presented in Table 3.7, correspond to a left or right shift of distribution from zero, regarding a the rate of increase (β_1) of a parameter.

Trends for Micronutrients Histograms for trends in micronutrient levels (carotenoids and vitamins) are presented below. Included are the carotenoids lutein, lycopene, α-carotene, β-carotene, β-cryptoxanthin, zeaxanthin and the vitamins: vitamin C, vitamin B6, vitamin A and vitamin E.

Seven of the ten histograms presented are right shifted from zero, indicating an overall positive trend. The trend for β-cryptoxanthin and vitamin A are shifted to the left, suggesting a decrease in the levels of these parameters. In histograms of the β_1 trends for zeaxanthin and vitamin E the largest bars centered around zero, thus indicating a little change.

Lutein Distribution of β_1 for lutein is shown in Figure 3.15; 95% of the individual β_1 values are shifted to the left. Highest β_1 correspond to individual rates of increase of over 0.03 μmol/L/month. The lowest values for β_1 were observed at -0.01μmol/L/month.

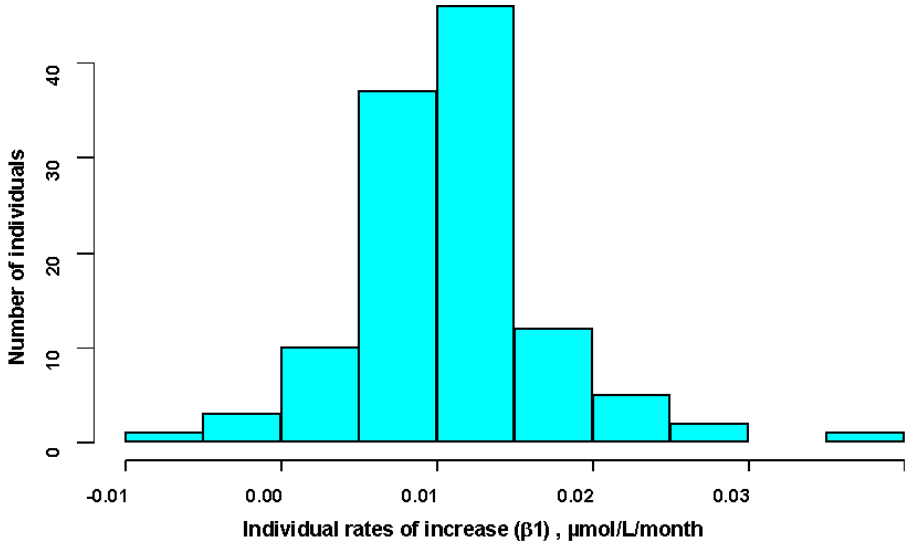

Figure 3.15: Distribution of individual rates of increase (β_1) for lutein.

Lycopene In the case of lycopene (Figure 3.16), the largest bar is at about 0.02 μmol/L/month (about 45 volunteers). Interestingly, several extreme values for β_1 were observed. β_1 values of up to 0.15 μmol/L/month (ten times higher than the majority) were in contrast to β_1 values of -0.10 μmol/L/month, remarkably lower (ten fold lower) than the average.

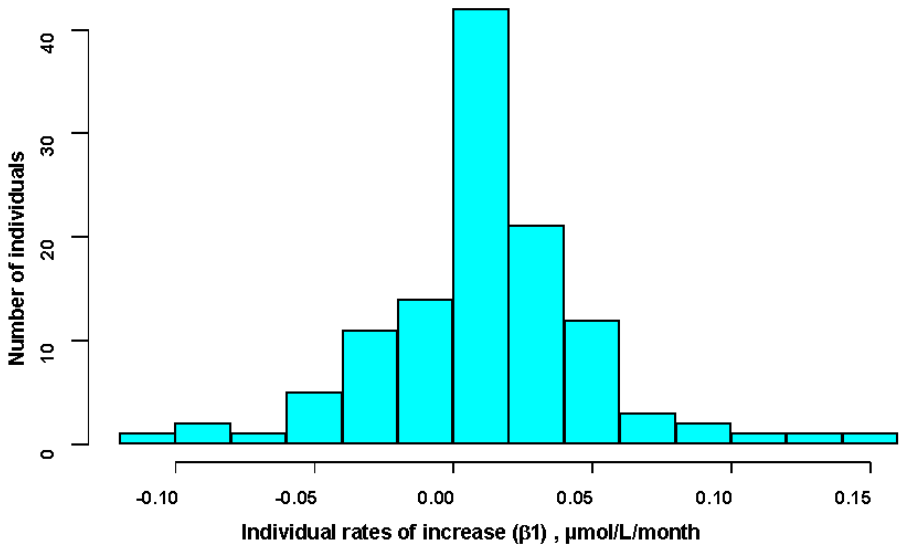

Figure 3.16: Distribution of individual rates of increase (β_1) for lycopene.

α-Carotene The histograms for α- and β-carotene, two structurally related carotenoids, are displayed in Figures 3.17 and 3.18.

In the case of α-carotene, a right shift in the distribution of β_1 was seen in over 80 individuals. The majority of subjects had a β_1 between 0.005 and 0.04 μmol/L/month, with the highest values between 0.01 and 0.02 μmol/L/month. Very high β_1 values of this carotenoid were observed in 4 individuals with up to 0.08 μmol/L/month.

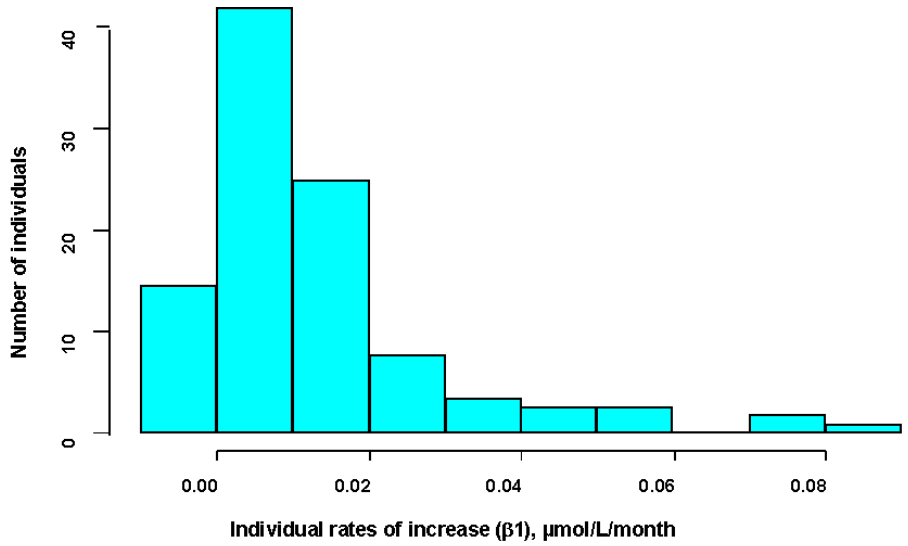

Figure 3.17: Distribution of individual rates of increase (β_1) for α-carotene.

β-Carotene β_1 values of β-carotene were significantly increased in almost 70% of the volunteers (see Fig. 3.18). The highest values of individual slopes for β-carotene were at 0.4 μmol/L/month.

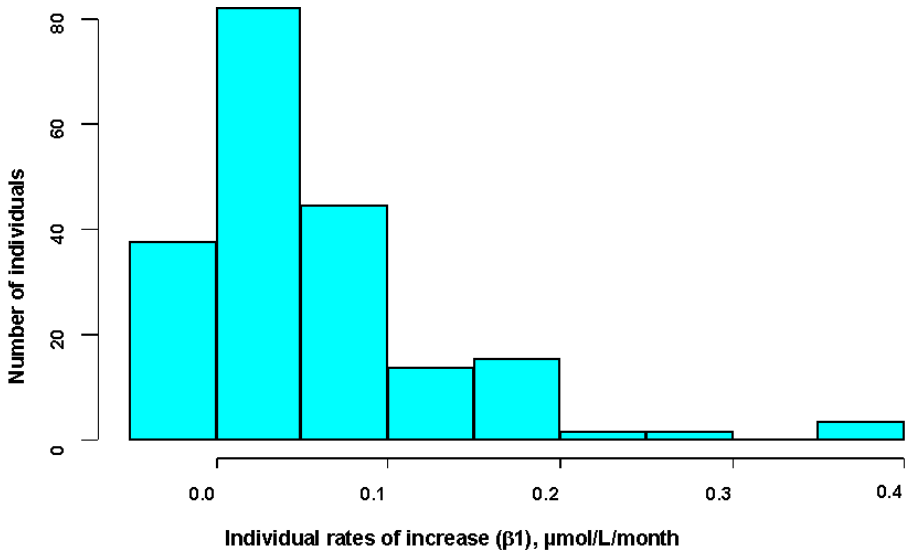

Figure 3.18: Distribution of individual rates of increase (β_1) for β-carotene.

β-Cryptoxanthin In contrast to other carotenoids β-cryptoxanthin shows a negative trend (Fig. 3.19). β_1 values of the majority of the study population (largest bars) is left shifted from zero.

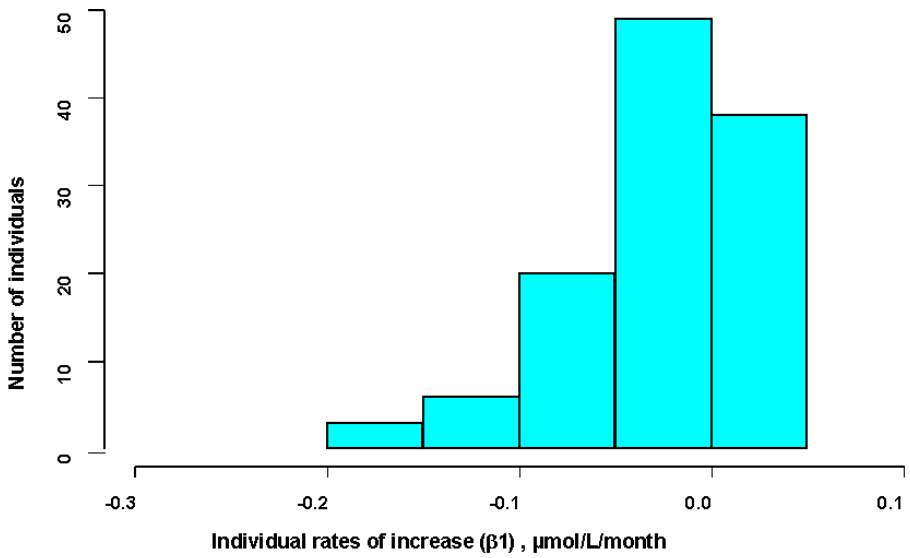

Figure 3.19: Distribution of individual rates of increase (β_1) for β-cryptoxanthin.

Zeaxanthin From the six carotenoids investigated zeaxanthin (Fig. 3.20) was the only one which did not show any significant change in blood levels reflected also in the individual rates of increase. This trend is also seen in the histogram, where the largest bars are centered around zero (representing almost 95% of the volunteers).

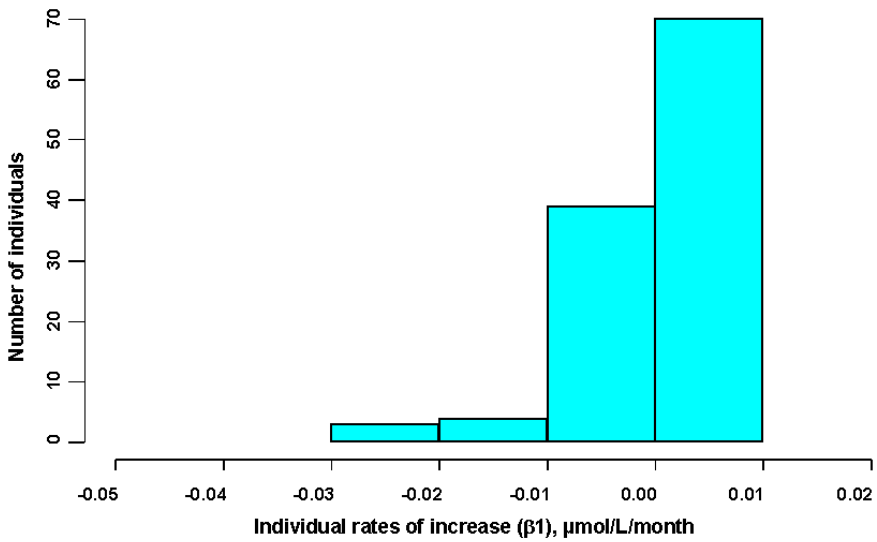

Figure 3.20: Distribution of individual rates of increase (β_1) for zeaxanthin.

Vitamin A, Retinol In the first approach using a descriptive statical analysis (see 3.1.1 in Table 3.3), it was observed that levels of vitamin A were slightly lower at the end of the study compared to base line (T0: 1.35 $\pm$ 0.35, T3: 1.33 $\pm$ 0.30). This was confirmed by the negative trend obtained from the longitudinal analysis where β_1 was -0.003 $\pm$ 0.006 μmol/L/month (see also Table 3.7). It can be taken from Figure 3.21 that the β_1 of the largest population of volunteers is shifted to the left, indicating an apparent decrease of this vitamin.

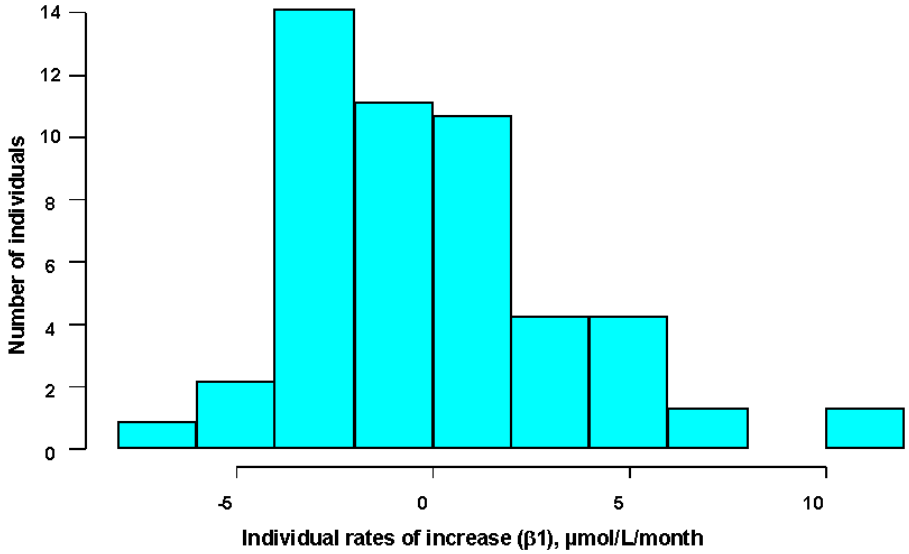

Figure 3.21: Distribution of individual rates of increase (β_1) for vitamin A (retinol).

Vitamin E, α-Tocopherol For vitamin E (α-tocopherol), it was observed that the β_1 values for about 90 individuals are around zero. The general β_1 was 0.08 ± 0.21 μmol/L/month and correlates with the distribution of individual β_1 between zero and 0.1 μmol/L/month. Few cases are observed with extreme high and low values.

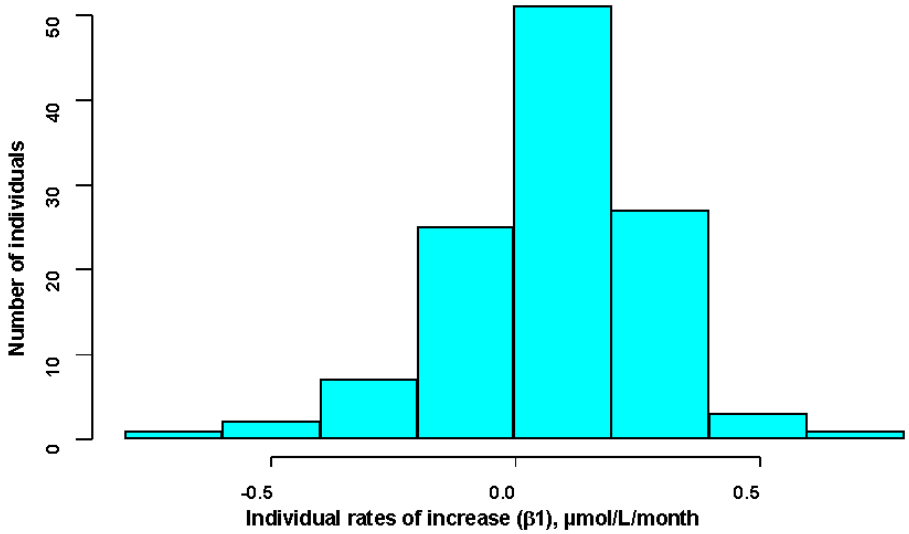

Figure 3.22: Distribution of individual rates of increase (β_1) for vitamin E (α-tocopherol).

Vitamin C As it can be seen in the histogram for Vitamin C (Fig. 3.23), that over 90 volunteers showed a positive trend, which reached values of β_1 of up to 6 μmol/L/month. Most of the individuals had a β_1 of about 2.0 μmol/L/month. Extreme low values for β_1 are observed at -4 μmol/L/month, which refers to only 2 individuals.

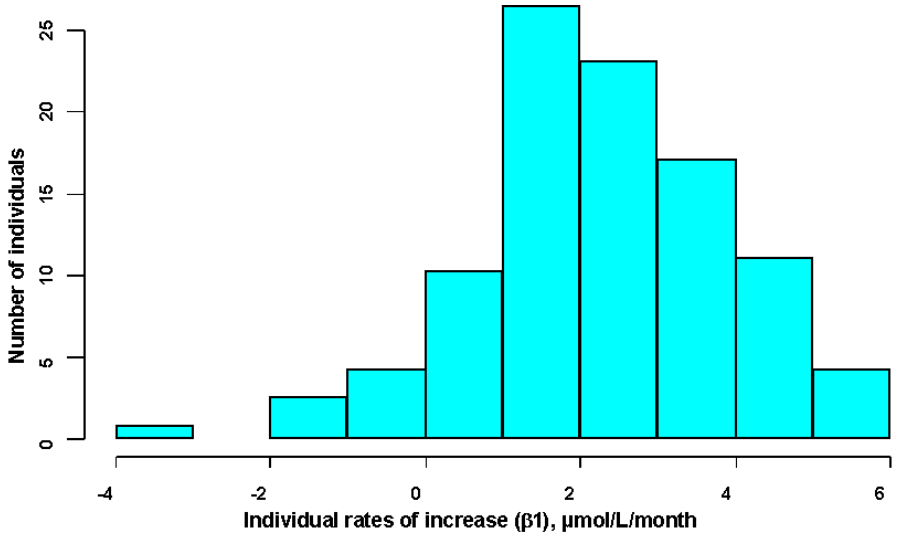

Figure 3.23: Distribution of individual rates of increase (β_1) for vitamin C.

Vitamin B6 As shown in the histogram for Vitamin B6 (Fig. 3.24) approx. 7 volunteers show a negative β_1 of this vitamin. About the same amount of individuals showed a remarkable increase of over 10 nmol/L/month. Approximately 80 volunteers had a monthly increase of over 2 nmol/L/month.

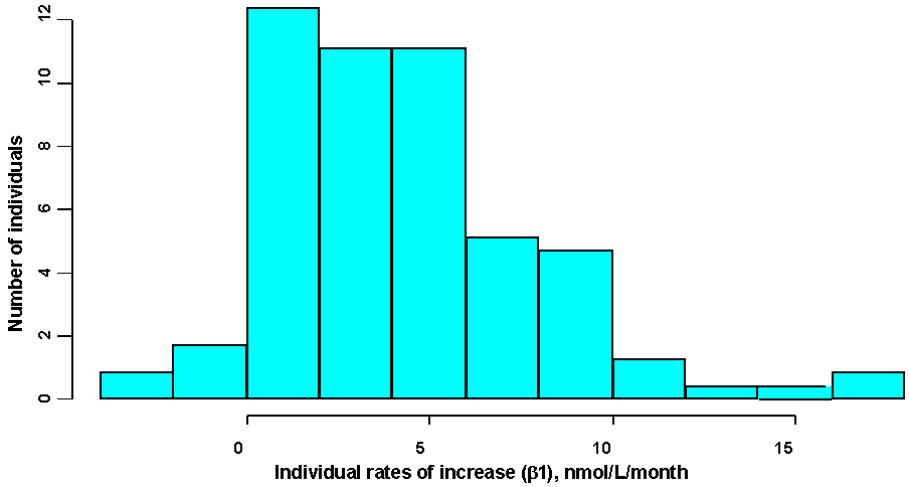

Figure 3.24: Distribution of individual rates of increase (β_1) for vitamin B6.

Biomarkers of Oxidative Stress Histograms for protein carbonyls, MDA and for homocysteine, are shown in the Figures 3.25, 3.26 and 3.27, respectively.

Protein Carbonyls The β_1 values are around zero. 2 subjects on the left arm of the histogram showed notably higher values than the rest of the population This single cases do not influence the average of β_1.

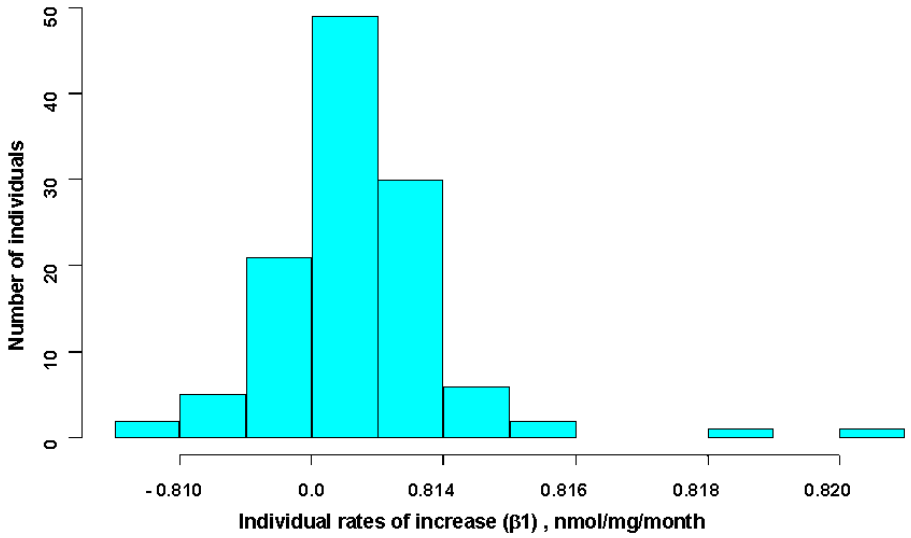

Figure 3.25: Distribution of individual rates of increase (β_1) for protein carbonyls.

MDA Figure 3.26 shows the distribution of the individual rates of increase of MDA. The largest two bars of the histogram are centered around zero. The trend of the individual rates of increase did not change significantly. There were however, about 12 cases with negative values. These negative values were (between -0.10 μmol/L/month and -0.025 μmol/L/month) about a ten fold lower than the average individual β_1, and represents less than 10% of the study population.

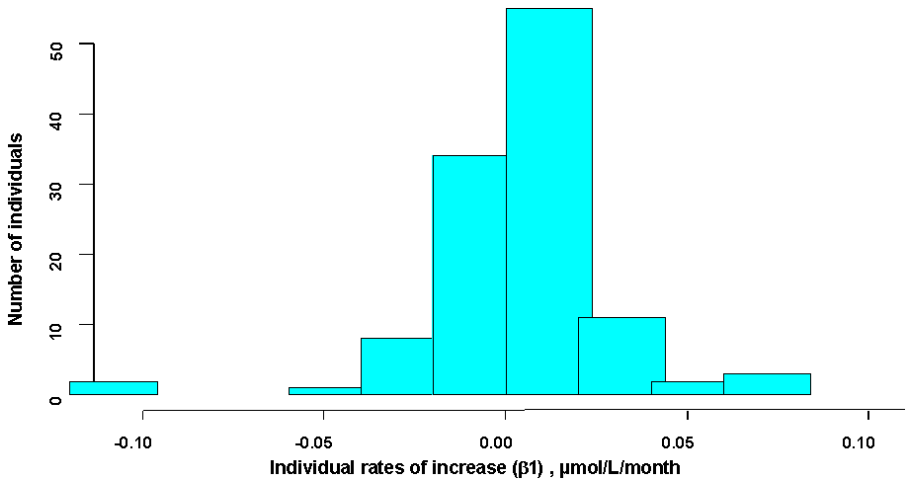

Figure 3.26: Distribution of individual rate of increase (β_1) for MDA.

Homocysteine Distribution of $\beta 1$ values of homocysteine are presented in Figure 3.27. The β_1 values for the entire study population of individuals are distributed around zero.

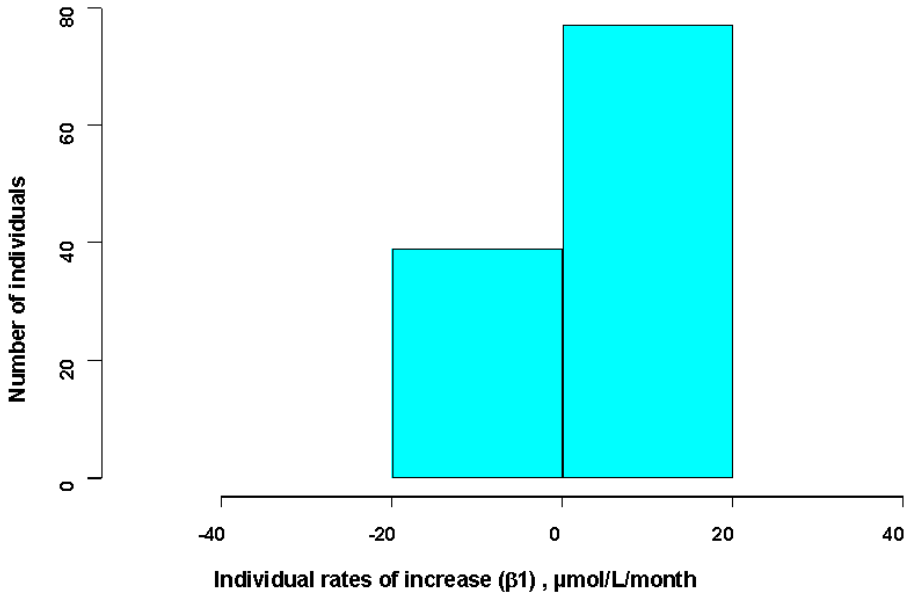

Figure 3.27: Distribution of individual rates of increase (β_1) for homocysteine.

Uric Acid Using the linear growth statistical model it was found that levels of uric acid had changed significantly during the study. As seen in the histogram,

values are shifted to the right, showing thus a positive trend in the values of β_1. The diagram has almost a symmetrical shape with few cases at both arms of the histogram, lowest values are found at 0.217 mg/dL/month and highest at 0.220 mg/dL/month. The rate of increase in most of the volunteers is between 0.217-0.219 mg/dL/month.

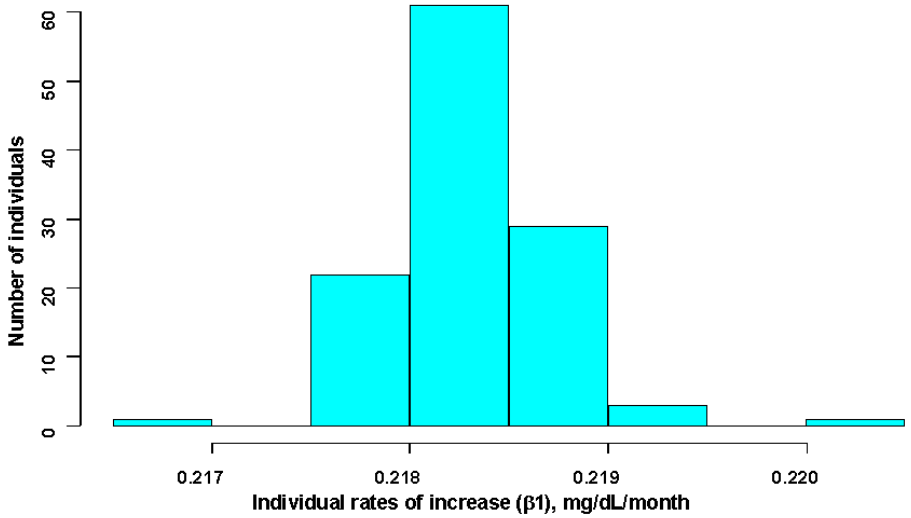

Figure 3.28: Distribution of individual rates of increase (β_1) for uric acid.

Clinical Parameters

Total Cholesterol The distribution of β_1 values are right shifted from zero. No β_1 values with a negative trend were observed. The major distribution of the individual rates of increase (β_1) was between 9.0 and 13.0 mg/dL/month, corresponding to almost 90 individuals. Only 2 individuals showed notably lower values in their β_1 compared to the rest of the study population.

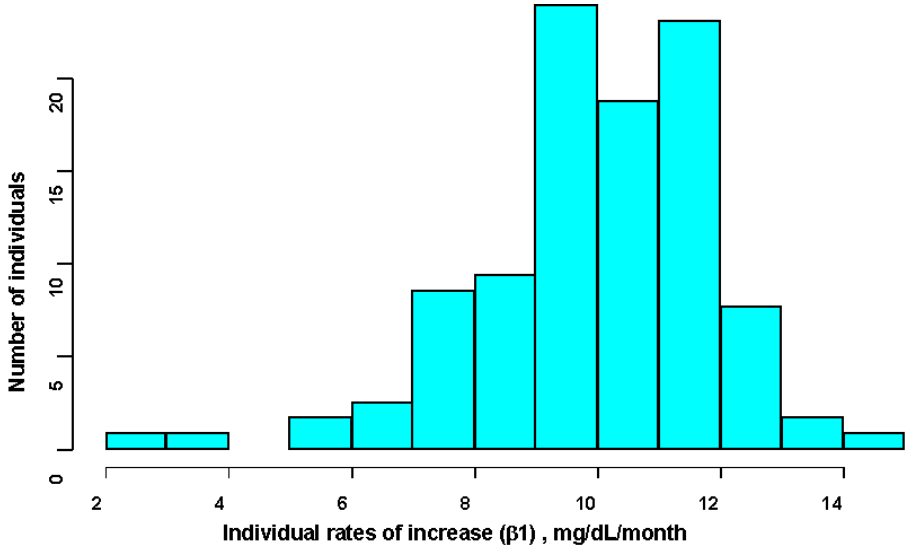

Figure 3.29: Distribution of individual rates of increase (β_1) for total cholesterol.

LDL-Cholesterol The distribution of β_1 values of LDL-cholesterol are right shifted from zero. No β_1 values with a negative trend were observed. The major distribution of the individual rate of increase (β_1) was between 5.0 and 8.0 mg/dL/month, corresponding to approx. 100 subjects. Only 1 individual showed a notably higher value in its β_1 compared to the study population.

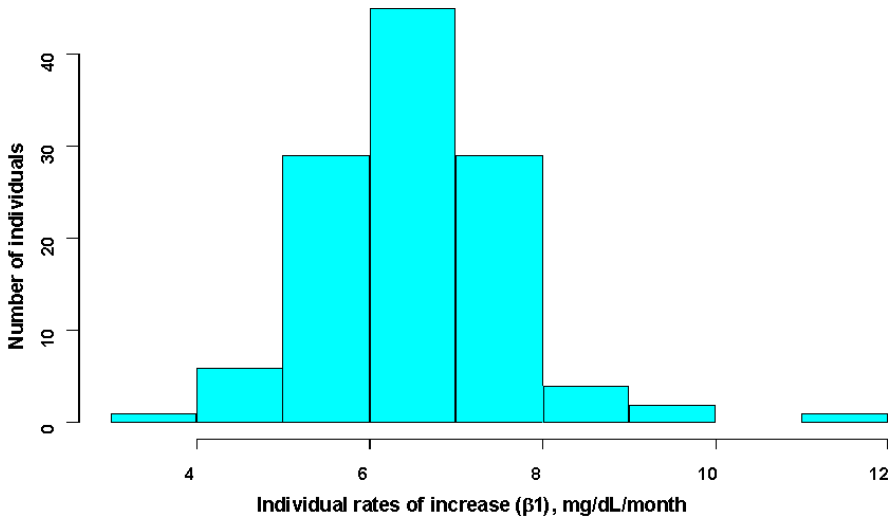

Figure 3.30: Distribution of individual rates of increase (β_1) for LDL-cholesterol.

HDL-Cholesterol The distribution of β_1 values of HDL-cholesterol are right shifted from zero. This positive effect was observed between 2.0 and 5.0 mg/dL/month, corresponding to approx. 100 subjects. Two individuals showed notably higher values in their β_1 compared to the rest of the study population (β_1 over 7.0 mg/dL/month).

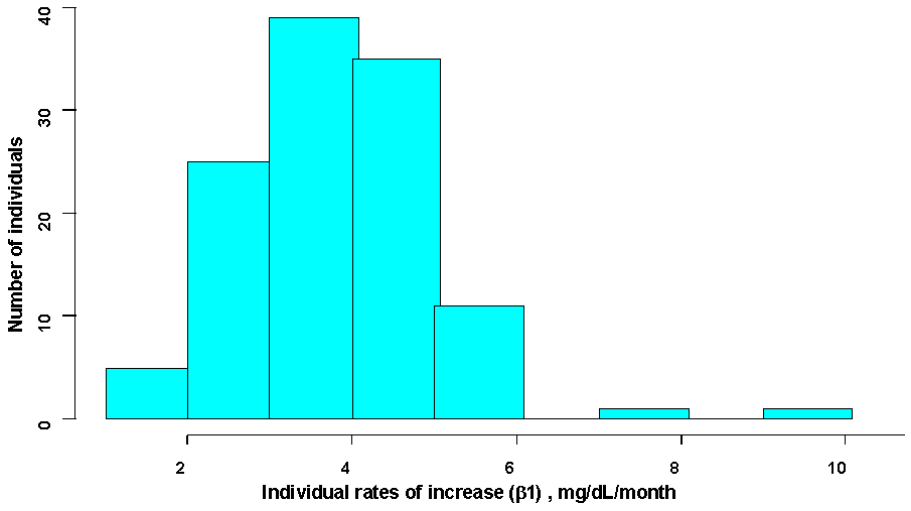

Figure 3.31: Distribution of individual rates of increase (β_1) for HDL-cholesterol.

Triglycerides The distribution of β_1 values of triglycerides are right shifted from zero. For more than 80 individuals β_1 is between 8.0 and 12.0 mg/dL/month.

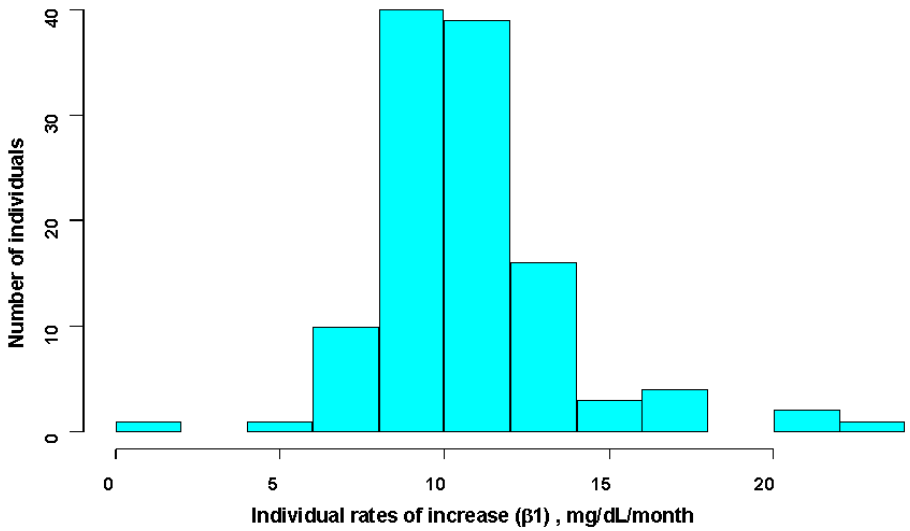

Figure 3.32: Distribution of individual rates of increase (β_1) for triglycerides.

Body Mass Index The histogram in Figure 3.33 shows the distribution of β_1 of BMI. Note that the main columns are around zero, demonstrating that no shift in BMI occurred during the study. There were 13 individuals with a $\beta_1 <$ -0.5, indicating weight loss. Six individuals however had a $\beta_1 > 0.5$, indicating gain of weight.

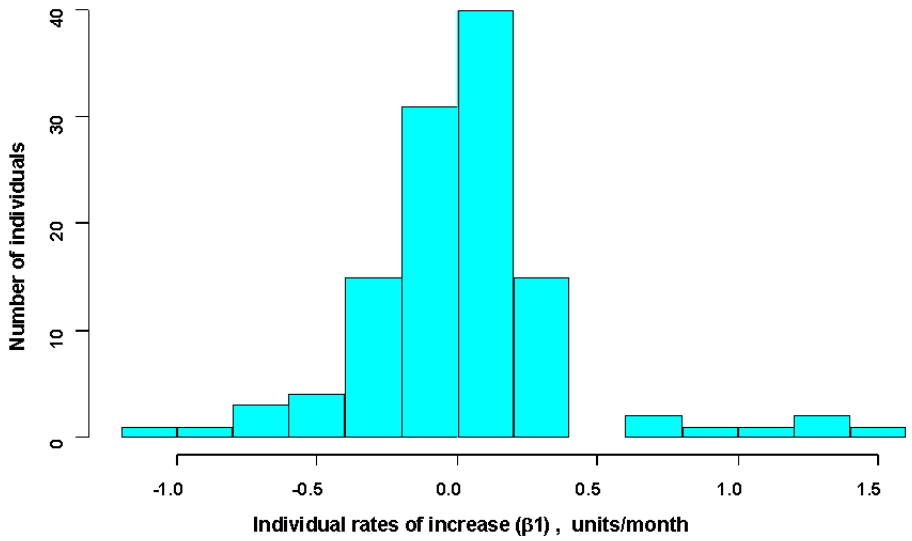

Figure 3.33: Distribution of individual rates of increase ($\beta 1$) for BMI.

Correlations Between Parameters Using Pair Plot analysis The correlation of two parameters can be analyzed by pair plotting their individual β_1 in a single diagram called pair-plot diagram (PPD, presented in Figure 3.34). The PPD is a multivariant visualization technique. Arranged on the diagonal of the PPD, some of the previously presented histograms are displayed. As an example Figure 3.34 presents how vitamin A, vitamin B6, vitamin C, α-carotene, β-carotene and cholesterol are correlated.

The upper and lower diagonal panels describe the relationship between the parameters. The upper diagonal panel B1 and the lower diagonal panel A2, are inverse to each another.

Panel B1 (or A2), shows the correlation between the rate of increase of vitamin A and vitamin B6. No correlation was found between the rate of increase of the lipid soluble vitamin A and the water soluble vitamin B6. No correlation was found between vitamin A and vitamin C (panel C1).

An interesting finding is that the water soluble vitamins (vitamin B6 and vitamin C), which showed significant higher values at the end of the study in the descriptive analysis, and significant rates of increase in linear growth statistical model, are not correlated to one another.

The structurally related compounds α-and β-carotene which showed significant higher values at the end of the study in the descriptive analysis, and significant rates of increase in the linear growth statistical model, were found to be correlated to one another (panel E4), but not correlated to vitamin A (panel E1), and not to the water soluble vitamins (vitamin C and vitamin B6).

Total cholesterol was found not to be correlated with the water soluble vitamins: vitamin B6 (panel F2), vitamin C (panel F3), nor with α-and β-carotene (panels F4 and F5, respectively). A slight correlation was observed between total cholesterol and vitamin A.

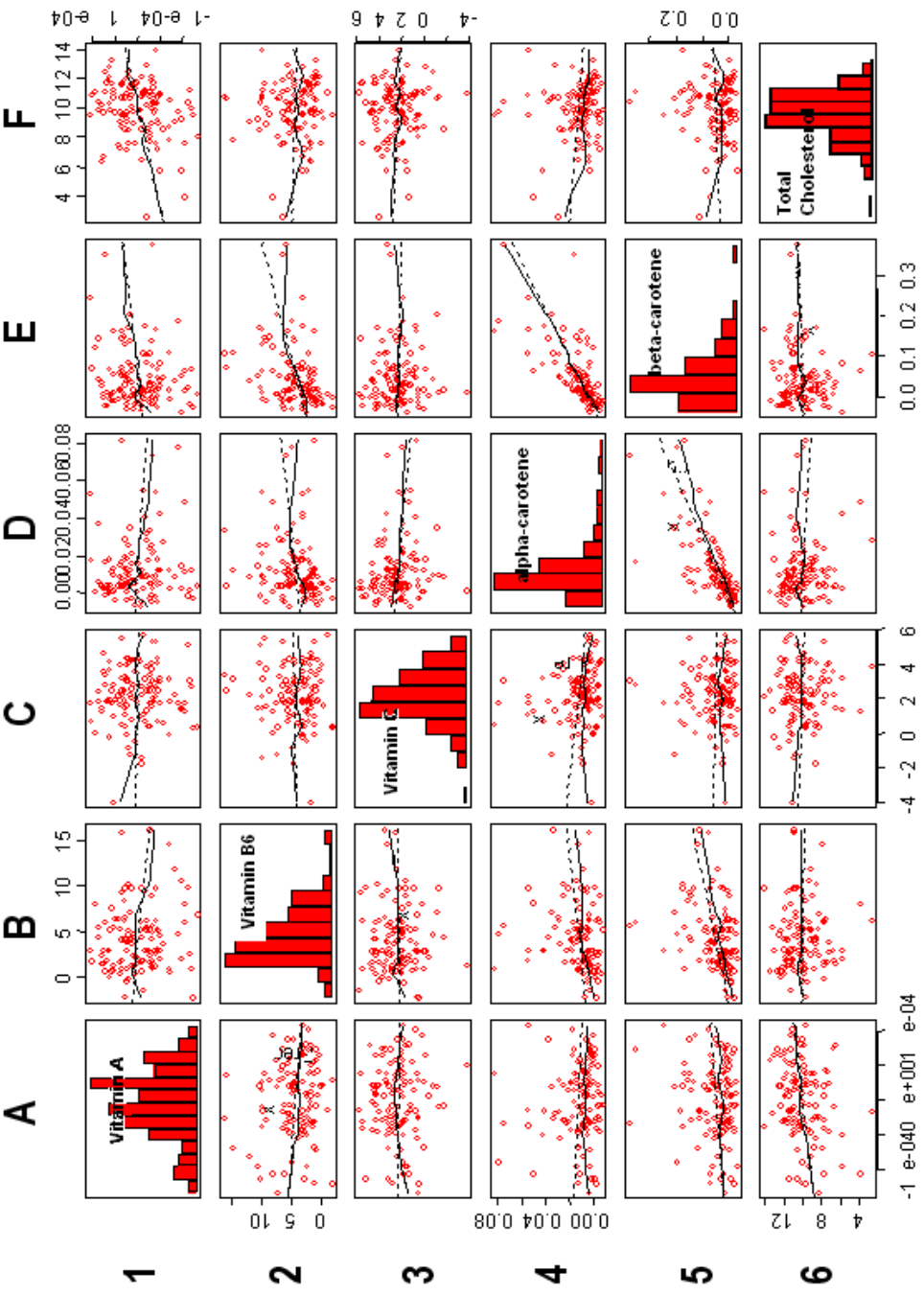

Figure 3.34: Pair plots showing correlations between β_1-values of analytes.

3.1.6 Compliance During the Intervention Study and Correlation of Micronutrient and Vitamin Levels to Specific Food Items

The increase in the vitamin and micronutrient levels can be explained in two ways. Firstly, the adherence of the volunteers to the program: compliance of taking five portions of fruit and vegetables a day should lead to higher levels of micronutrients and vitamins.

Secondly, selection of specific food items consumed (recorded through the food frequency questionnaire) rich in specific antioxidants/vitamins, may explain the higher levels of a specific micronutrient.

Compliance Compliance for fruit and vegetable intake in the food frequency questionnaire (FFQ) was assessed in the frequency categories: never, less than once a week, about once a week, several times a week, about once a day and several times a day. The overall frequency index has a total possible score ranging from 0 to 32.

The resulting score was reassigned to one of the three categories of the nutritional state: A=optimal (score grater than 16), B=normal (score between 14 to 16 points) and C=poor nutritional state (score less than 13 points). A, B and C refer to the current nutritional state of an individual at a certain time point or time period. Table 3.8 shows the percentages of volunteers according to their nutritional state at the 7 evaluated food frequency questionnaires (FFQ).

At the beginning of the study, 66.7% of the study population had an optimal nutritional state. The percentage of volunteers with a poor nutritional state (25%) was higher than the volunteers with a normal nutritional state (8.3%). Already at the second week of the intervention study (corresponding to the second FFQ), the percentage of volunteers with a poor nutritional state (C)was down to 7.1%. Percentage of volunteers with a normal nutrition (B) decreased as well (3.1%). At this time point, almost 90% of volunteers had acquired an optimal nutritional state. The percentages observed at the time point of the second FFQ stayed more or less around the same values, except at the last time point (end of the study). At the end of the study, 94% of the volunteers were in an optimal nutritional state, about

Table 3.8: Nutritional States (7 evaluated FFQ). A=optimal, B=normal and C=poor

	Nutritional State		
FFQ	C	B	A
1	25.0%	8.3%	66.7%
2	7.1%	3.1%	89.8%
3	7.3%	5.5%	87.2%
4	10.7%	9.8%	79.5%
5	9.8%	6.9%	83.3%
6	9.5%	8.6%	81.9%
7	1.9%	3.9%	94.2%

4% in category B, and about 2% of volunteers had a poor nutritional state.

Compliance, Fresh Fruit Intake Specific intake of fruit was further assessed by grouping all kinds of fruit items in the food frequency questionnaire under the common name of "fresh fruit". Scoring from fresh fruit frequency intake was assigned to one of the three categories of the nutritional state: A=optimal (score grater than 16), B=normal (score between 14 to 16 points) and C=poor nutritional state (score less than 13 points).

Table 3.9 shows the percentages of volunteers according to their frequency in fresh fruit intake; evaluated with the 7 food frequency questionnaires (FFQ).

At the beginning of the study, 69.4% of the study population had an optimal fresh fruit intake. The percentage of volunteers with a normal fresh fruit intake (25%) was higher than the volunteers with a poor fruit intake (5.6%). Already at the second week of the intervention study the percentage of volunteers with an optimal fresh fruit intake (A) went up to 94.3%. The percentages observed at the time point of the second FFQ stayed more or less around the same values for the rest of the study. The highest percentages of volunteers with either a normal or an optimal fruit intake was observed at the 6th FFQ, B=4.9% and A=94.3%. At the end of the study, 91.4% of the volunteers had an optimal fruit intake, about 5% were in category B, and about 3.4% of volunteers had a poor fresh fruit intake.

Table 3.9: Frequency in fresh fruit intake (7 evaluated FFQ). A=optimal, B=normal and C=poor

	Nutritional State		
FFQ	C	B	A
1	5.6%	25.0%	69.4%
2	1.6%	4.1%	94.3%
3	2.4%	3.3%	94.3%
4	3.3%	4.9%	91.9%
5	3.4%	4.2%	92.4%
6	0.8%	4.9%	94.3%
7	3.4%	5.2%	91.4%

Compliance, Vegetable Intake Specific intake of vegetables was further assessed by grouping all vegetable items (cooked and/or fresh) included in the food frequency questionnaire under the common name of "vegetables". Scoring from vegetable frequency intake was assigned to one of the three categories of the nutritional state: A=optimal (score grater than 16), B=normal (score between 14 to 16 points) and C=poor nutritional state (score less than 13 points).

Table 3.10 shows the percentages of volunteers according to their frequency in vegetable intake; evaluated with the 7 evaluated food frequency questionnaires (FFQ).

At the beginning of the study, 27% of the study population had an optimal vegetable intake. The same percentage was observed for individuals with a poor vegetable intake. 46% of the volunteers had a normal vegetable intake.

At the second week of the intervention study the percentage of volunteers with an normal vegetable intake (B) went down only by 3%, and did not even double for individuals with an optimal intake (A). The percentages observed throughout the FFQ stayed between 43% and 51% for individuals in category A, between 34% and 45% for individuals in category B, and between 12% and 15% for individuals in category C. At the end of the study, 49% of the volunteers had an optimal vegetable intake, about 34% were in category B, and about 17% of volunteers had a poor vegetable intake.

Table 3.10: Frequency in vegetable intake (7 evaluated FFQ). A=optimal, B=normal and C=poor

	Nutritional State		
FFQ	C	B	A
1	27.0%	46.0%	27.0%
2	14.2%	43.3%	42.5%
3	12.4%	36.4%	51.2%
4	11.7%	40.0%	48.3%
5	14.8%	40.2%	45.1%
6	11.8%	45.4%	42.9%
7	17.1%	34.2%	48.7%

Table 3.11: Self-estimated compliance to portion intake. YES= 5 or more fruit and vegetable portions were consumed. NO=did not consumed 5 or more portions.

	Self-Estimated Compliance	
FFQ	NO	YES
1	5.9%	94.1%
2	1.7%	98.3%
3	2.5%	97.5%
4	1.7%	98.3%
5	1.7%	98.3%
6	0%	100%
7	0.9%	99.1%

Compliance, Portion intake Portions of fruit and vegetable was desired to be 5 or more. Volunteers were asked to self-estimate their compliance to the aimed amount of portions by answering with "YES" (**did** take 5 portions or more) or "NO" (**did NOT** take 5 portions or more). Table 3.11 shows the results of the self-estimated portion compliance at the 7 food frequency questionnaires time points.

Volunteers were generally stating to have complied to ingest 5 portions of fruit and vegetable. Interesting observation is at FFQ=1 were already 94.1% of volunteers claim to be doing 5 a day. Already at the time of the second FFQ, self-estimated

compliance is almost perfect. At FFQ=6, self-estimated compliance is 100%

Compliance. Migration Model Compliance was also analyzed dynamically by comparing two intervention periods. The first period (Pd-1) was defined as: base line to 15-days. The second period (Pd-2) was from 15-days to end of the study (section 2.3.6). Volunteers' compliance was assessed with a dynamic migration model. The migration model analyzes frequencies of change between nutritional states (A, B, C) within to intervention periods (Pd-1, Pd-2).

Figure 3.35 shows the probability (in percentages = P) of an individual to follow indicated migration patterns between Pd-1 and Pd-2. Migration patterns between periods were not statistically different from one another, indicating a stationary behavior of an individual. This means, once an individual adopted a certain nutritional state (A, B or C) in Pd-1, it was not likely that, in Pd-2, the same individual would migrate to a higher or lower nutritional state.

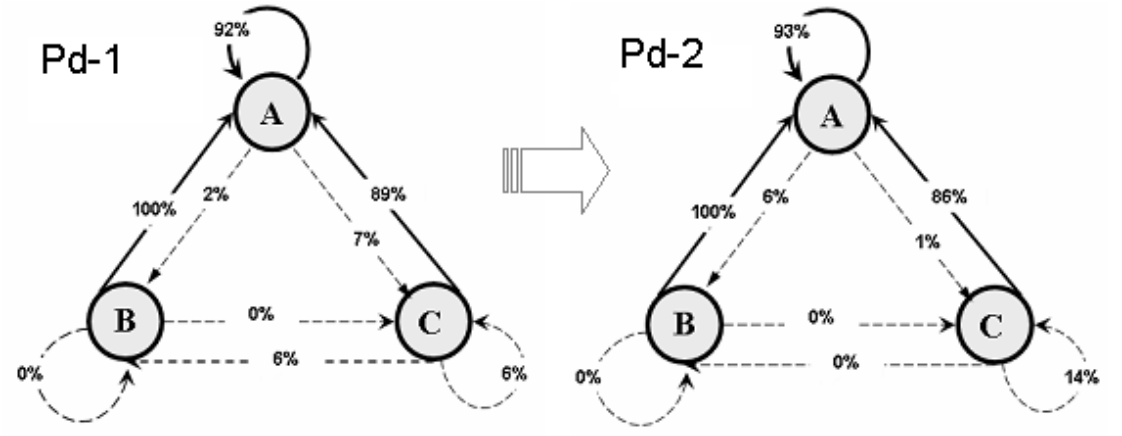

Figure 3.35: Schematic migration patterns. Dotted arrow: P $\leq$ 20%, thin arrow: 20%< P $\leq$ 40%, bold arrow: $P > 40\%$

For example, at Pd-1, the probability that a volunteer who was in the nutritional state A remains in this nutritional state, is 92%. Or, the probability that a volunteer migrates from C to A is 89%. At Pd-2, an individual found in A has a 93% probability to remain in A; or, the individual who had migrated from C to A has in Pd-2 an 86% probability to remain in A.

At Pd-1 and Pd-2, P=100% of individuals with a normal nutrition status migrated to an optimal nutritinal state (from B to A). It was observed that in the second intervention period (Pd-2), there was a 14% of probability that individuals with poor nutrition (C) would not migrate to a better nutritinal state in comparison to the first fifteen days of intervention where this probability was only 6%.

Food Intake Related Increase of Levels of Micronutrient An additional way to explain the observed increases in micronutrient levels, was to correlate the frequency in consumption of a specific food item with a specific micronutrient. Shown are only the diagrams of parameters which showed a clear correlation between plasma levels of micronutrients and certain food items.

Numbers found on the abscissa of the diagrams are the volunteers total scoring of the food item throughout the intervention study. The score number is arbitrary in units. Values found on the ordinate give the blood concentration.

Lutein The increased lutein levels observed in the study population was related to the consumption of cauliflower, broccoli, cabbage (stem and savoy cabbage, among others) and sprouts (item e15 of the FFQ). Figure 3.36 shows a positive trend for this carotenoids in relation to this item (e15). It can be seen that there are several out layers, mainly around scoring of 20 to 25 and with levels between 0.4 and -0.4 μmol/L/month. Out layers around scoring of 20 to 25 show a faster response in increasing lutein levels related to the consumption of this food item.

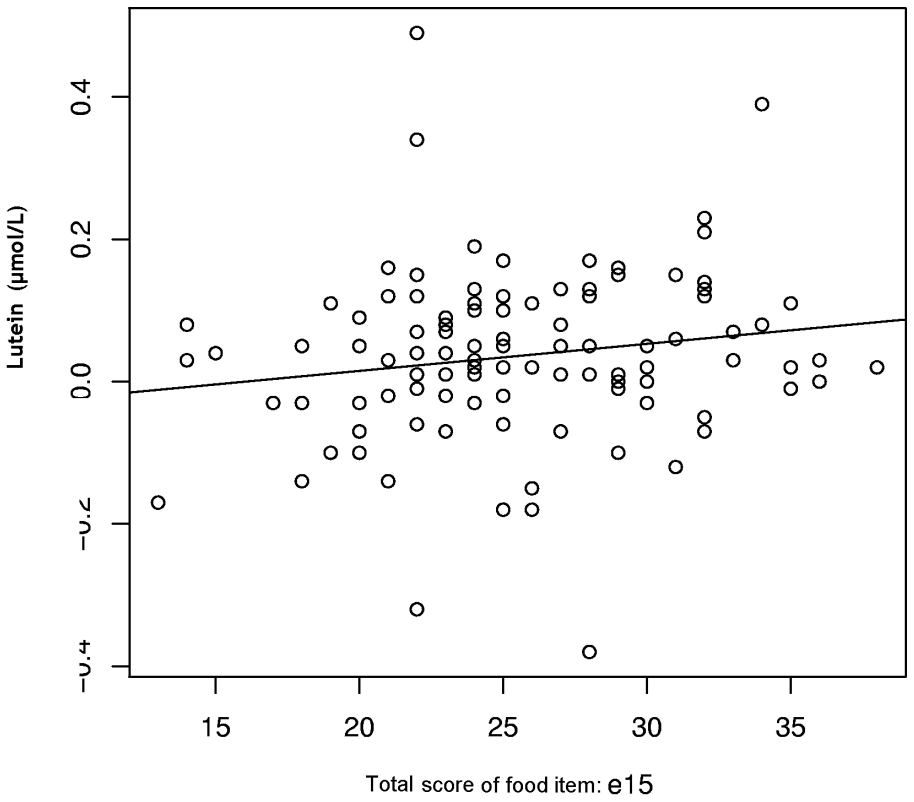

Figure 3.36: Lutein levels related to intake of cauliflower, broccoli, cabbage, stem cabbage, sprouts and savoy cabbage (e15)

Lycopene The increase of lycopene was attributed to more than one item. Fig. 3.37 shows that the consumption of fish (E4), celery, beet, spinach (leafy vegetables) (E11) and fruit juices (E42) is positively correlated to the increase of lycopene. The last of the four plots show, however, a negative trend for lycopene related to the consumption of multivitamin beverages (E43).

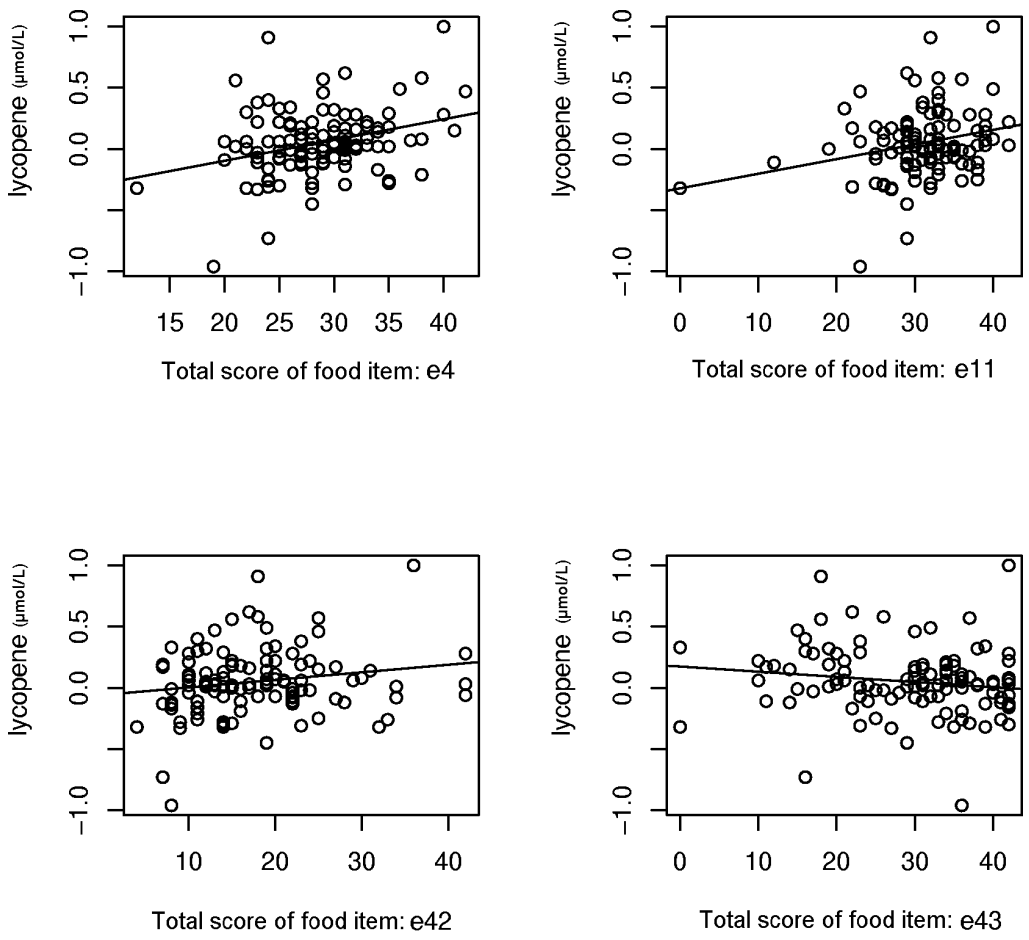

Figure 3.37: Lycopene levels related to intake of fish (e4), celery, beet, spinach and leafy vegetables (e11), fruit juices (e42) and multivitamin juices (e43).

α-Carotene In the case of α-carotene (Fig. 3.38) , the consumption of cooked vegetables (E20) was positively correlated to the levels of this micronutrient. Consumption of fruit juices (e42), was negatively correlated to levels of α-carotene. Figure 3.39 also shows a negative trend of this carotenoid related to the consumption of fish.

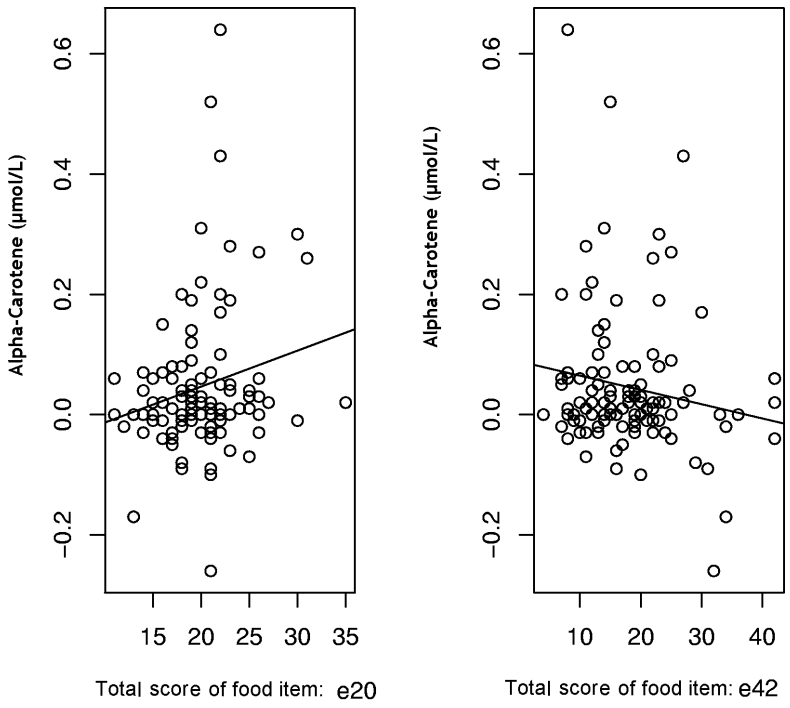

Figure 3.38: α-carotene levels related to intake of cooked vegetables (e20) and fruit juices (e42)

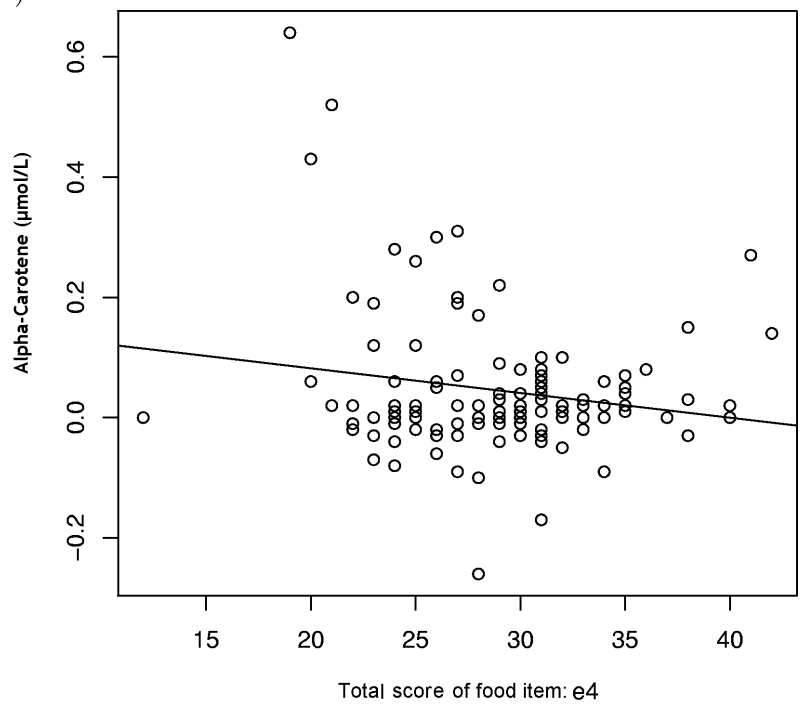

Figure 3.39: α-Carotene levels correlated to intake of fish (e4)

Vitamin B6 Vitamin B6 levels (Fig.3.40) were found to be positively correlated to the consumption of corn, oat, bran flakes and granola (e37), and negatively correlated to the consumption of tropical fruits: pineapple, kiwi, mango, maracuja (e27). In this last case oulayers found at a scoring of 10 to 20, showed values of up to 150 nmol/L.

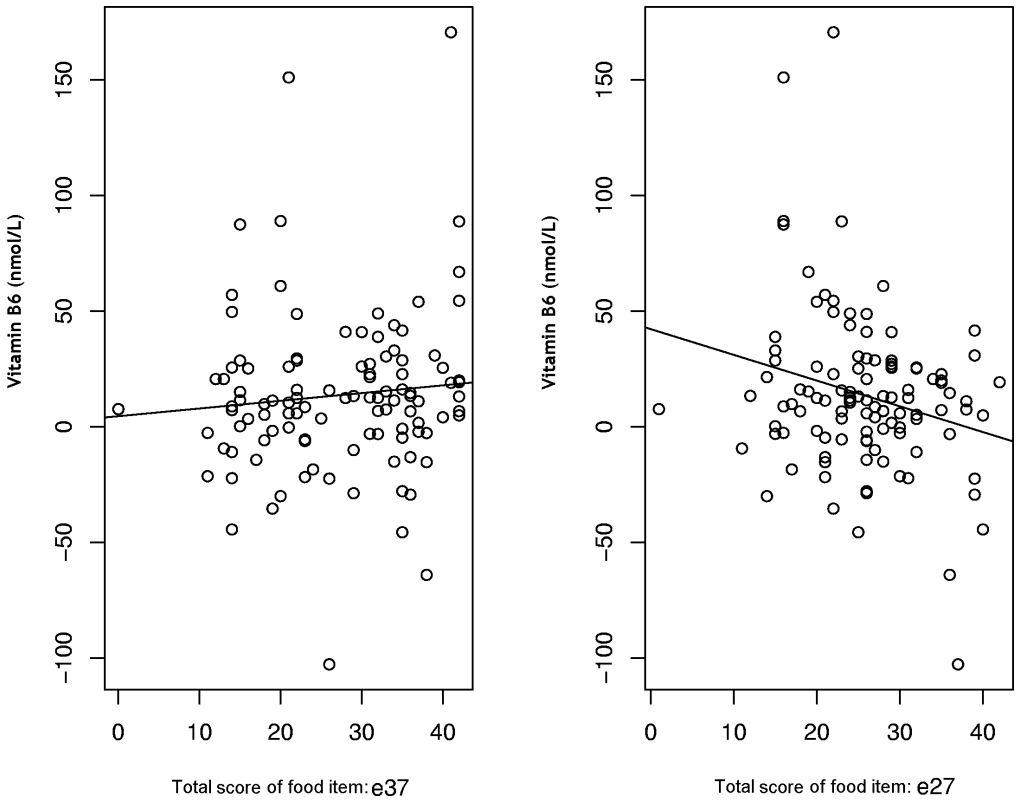

Figure 3.40: Vitamin B6 levels related to intake of corn, oat and bran flakes or granola (e37) and tropical fruits such as pineapple, kiwi, mango and maracuja (e27).

Vitamin C Figure 3.41 shows that the consumption of apples, pears, quinces (e24) is positively correlated to the increase of Vitamin C. Extreme values are seen at a scoring of over 35 at a concentration of about 60 μmol/L.

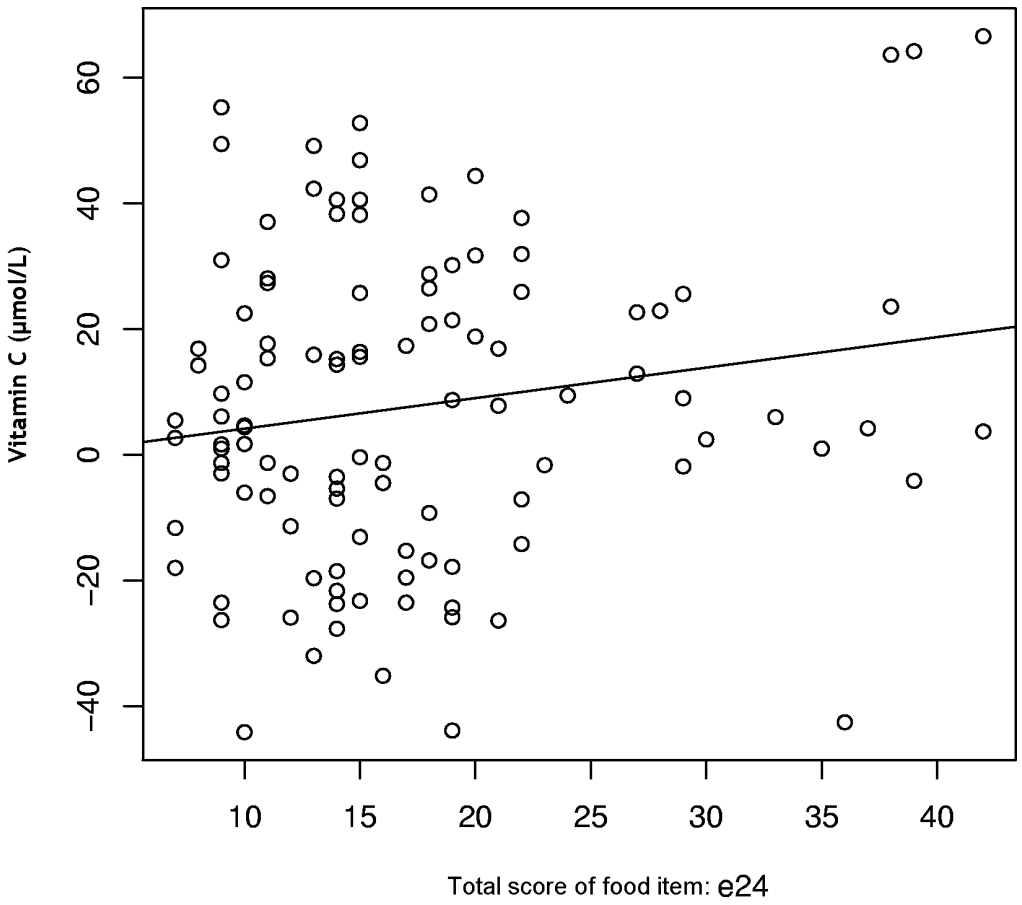

Figure 3.41: Vitamin C levels related to intake of fruits like apple, pears and quince (e24).

3.1.7 Summary of Compliance to the Intervention Study

The significant increased levels of micronutrients observed in the majority of volunteers can be explained in two ways. Firstly according to their compliance to the study (eating the desired five or more portions of fruits and vegetables). Subjects were classified according to their nutritional status, A=optimal, B=normal, C=poor nutritional behavior. Subjects who complied with the study migrated to a higher level of nutritional status. By remaining in an upper level, their levels of micronutrients increased. This migration effect was observed in the majority of

the people, pointing to a successful intervention and thus, explaining the observed increased levels of micronutrients.

Secondly, the increased levels of micronutrients can be correlated with the high consumption of a certain food item.

3.2 Liposome Model

Unilamellar liposomes, a lipid bilayer membrane (lipophilic compartment) with an aqueous core (hydrophilic compartment) resemble the membrane-cytosol structure of a cell and are therefore well suited to study membrane-bound antioxidants. Due to their lipophilic character carotenoids are readily incorporated into membranes. Anchored to membranes they are able to scavenge lipidperoxyl radicals protecting lipids in the membrane from lipid peroxidation and the cell from oxidative damage. From the "5 a Day" study, it was seen that levels of carotenoids in volunteer´s plasma increased. Higher levels of carotenoids acquired through the diet suggest an improved protection of tissues against oxidative damage. Since the various kinds of carotenoids are not accumulated as individual compounds and are therefore found in mixtures in tissues, synergistic antioxidant effect of these compounds have been suggested. Such possible synergistic antioxidant effects can be investigated using unilamellar liposomes labeled with mixtures of selected carotenoids.

The carotenoids lutein, zeaxanthin and lycopene were incorporated into liposome´s membranes as single compounds and in mixtures in order to analyze for possible synergisms.

Unilamellar Liposomes Loaded with Lutein and Lycopene To investigate whether an equimolar mixture of lutein and lycopene acts synergistically in *unilamellar* liposomes, three batches of these liposomes were prepared. Two batches, one with lutein (n=5) and one with lycopene (n=5) were prepared at a final concentration of 5 nmol carotenoid/mg lipid, a third batch (n=5) with 5 nmol carotenoid/mg lipid was an equimolar mixture of lutein and lycopene (2,5 nmol from each carotenoid). Batches were compared to a control, which was a batch of the same kind of liposomes without carotenoid load. Lipid peroxidation was initiated by incubation of the liposomes with AAPH (a water soluble radical initiator at a final concentration of 0,5 mM). Conjugated dienes are formed during the process of lipid peroxidation of the unsaturated fatty acids present in the liposome´s membrane and were chosen to follow the lipid peroxidation process (liposome decay) by measuring their absorbance at 234 nm. The diene concentra-

tion can be calculated from the absorbance and the molar extinction coefficient (ϵ = 2.8 10^4 L/mol * cm). Figure 3.42 shows the diene formation, given in nmol diene / mg lipid for the three different liposome preparations for a period of 120 min.

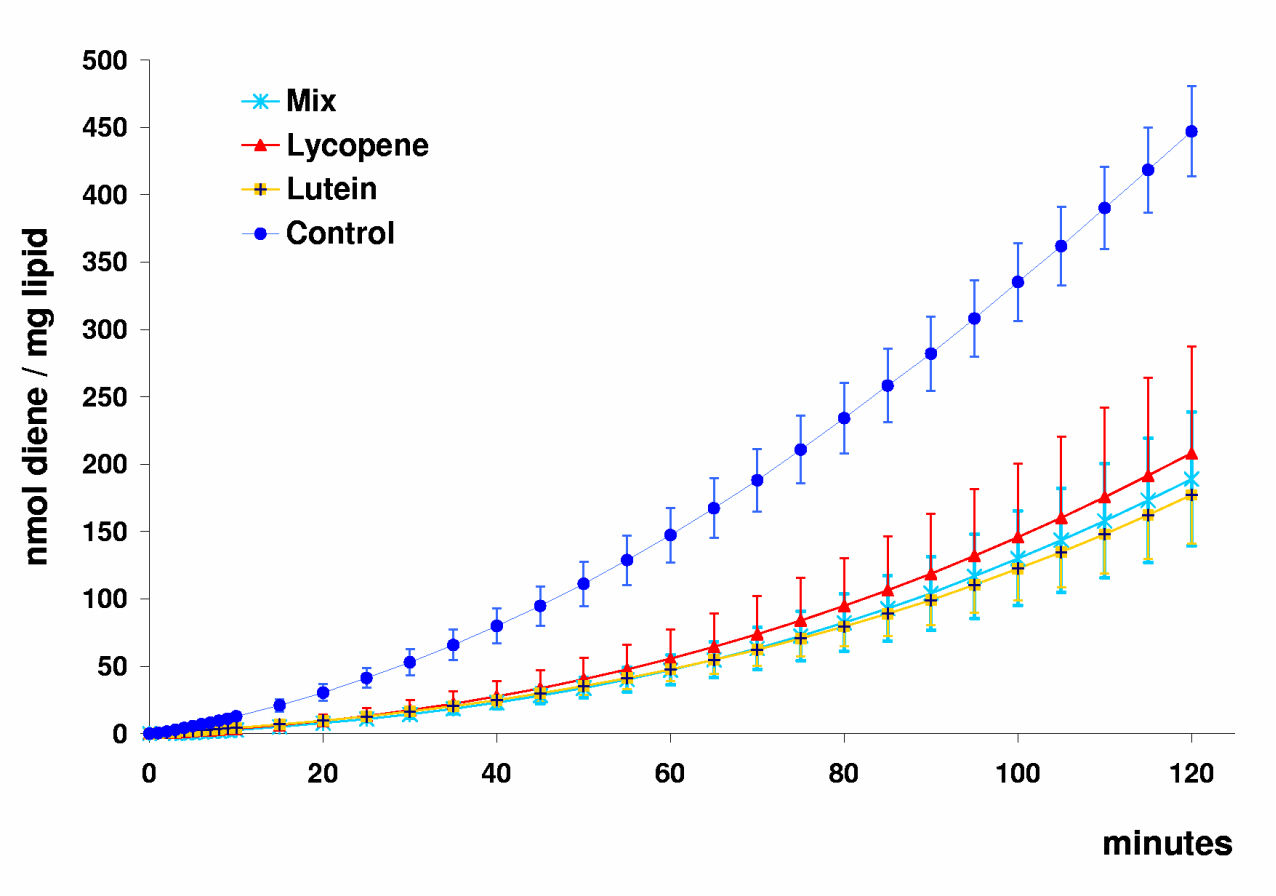

Figure 3.42: Unilamellar liposomes loaded with lycopene, lutein and an equimolar mixture of lutein and lycopene are challenged for lipid peroxidation (AAPH), and compared to the control. Diene formation, normalized for (nmol diene / mg lipid.) was measured as indicator of lipid peroxidation.

As a result of the lipid peroxidation initiated by AAPH the control liposomes reach levels of almost 500 nmol diene/mg lipid after 120 minutes. Carotenoids anchored to the liposome's membrane delay lipid peroxidation. Comparing the curves it can be seen that lutein loaded liposomes are some what better protected against lipid peroxidantion than the liposomes loaded with lycopene; less dienes are formed. At

120 min. about 180 nmol dienes/mg lipid are formed in liposomes loaded with lutein, whereas about 200 nmol dienes/mg lipid are formed in lycopene loaded liposomes, thus indicating lutein has a better antioxidant effect in this . From the curve of the equimolar mixture (lutein and lycopene at a final concentration of 5 nmol carotenoid/ mg lipid) it can be seen that diene formation is about 190 nmol diene/mg lipid at t=120 min. This indicates an additive antioxidant effect of an equimolar mixture of lutein and lycopene in unilamellar liposomes, no synergism.

Unilamellar Liposomes Loaded with Lutein and Zeaxanthin The predominant carotenoids of the macular pigment are lutein and zeaxanthin. Although only lutein levels were significantly increased in the intervention study, an equimolar mixture of lutein and zeaxanthin was also tested for possible synergistic antioxidative effect in the liposomal system. Batches (n=3) of liposomes loaded with these carotenoids were prepared to yield a final concentration of 1 nmol carotenoid/ mg lipid. Figure 3.43 shows the antioxidant effect of lutein, zeaxanthin and their mixture. For the mixture of lutein and zeaxanthin in the liposomes, 0.5 nmol carotenoid /mg lipid were mixed for a final concentration of 1 nmol carotenoid mixture/ mg lipid.

Diene formation initiated by AAPH in liposomes reached almost 150 nmol diene/mg lipid after 120 minutes. Comparing the curves showing the antioxidant effect of the carotenoids tested, it can be seen that zeaxanthin loaded liposomes are better protected against lipid peroxidation than the liposomes loaded with lutein. At 120 min. about 110 nmol dienes/mg lipid are formed in liposomes loaded with lutein, whereas only about 90 nmol dienes/mg lipid are formed in zeaxanthin loaded liposomes, thus indicating zeaxanthin is better antioxidant in this system. From the curve obtained with an equimolar mixture of lutein and zeaxanthin (at a final concentration of 1 nmol carotenoid/ mg lipid) it was found that diene formation at 120 min. is about 100 nmol diene/mg lipid. This corresponds to the theoretical additive value calculated as the sum of dienes formed from liposomes loaded with lutein and zeaxanthin at a concentration of 0.5 nmol carotenoid/mg lipid.

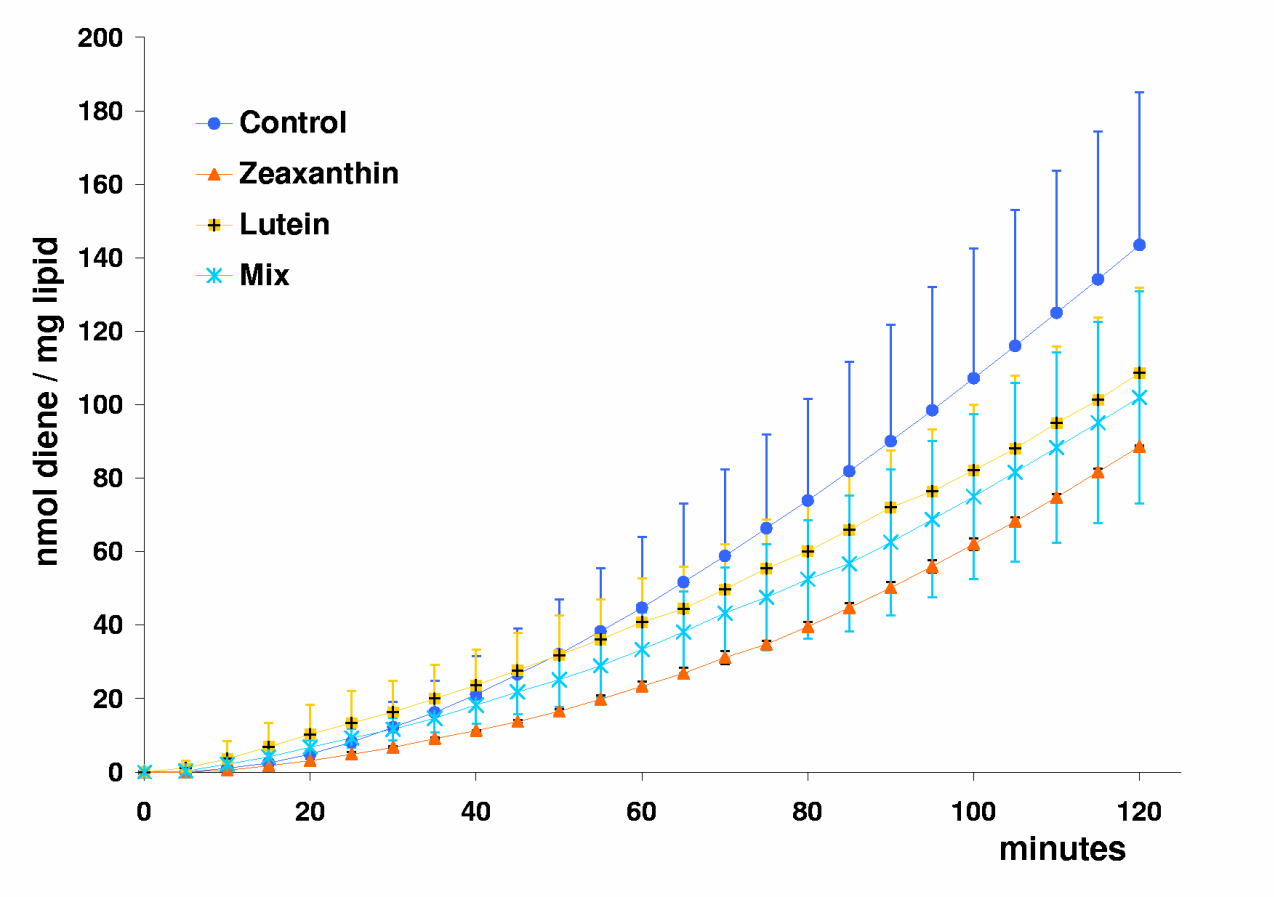

Figure 3.43: Unilamellar liposomes loaded with zeaxanthin, lutein and an equimolar mixture of lutein and zeaxanthin are challenged for lipid peroxidation (AAPH) and compared to the control. Diene formation (nmol diene / mg lipid) was measured as indication of lipid peroxidation.

3.2.1 Summary of the Study of Unilamellar Liposomes Loaded with Carotenoids

Unilamellar liposomes were used to study the effects of combinations of antioxidants in a membrane model. The antioxidant effect of the equimolar mixtures of lutein and zeaxanthin, lycopene and lutein is additive. The following table presents the relative formation of conjugated dienes at t= 120 min. The equimolar mixture of carotenoids in liposomes had the same final concentration (mg carotenoid/mg

lipid) as the carotenoids measured individually. Therefore, the theoretical additive antioxidant effect of liposomes loaded with an equimolar carotenoid mixture, was defined as the sum of the dienes formed from the single carotenoid-loaded liposomes divided by two.

	conjugated dienes (nmol/mg lipid) at 120 min.		
Components	Theoretical	Measured	SD (±)
Control	-	143.5	41.6
Lutein	-	108.7	23.1
Zeaxanthin	-	88.64	0.4
Mixture	98	101.9	28.8
	conjugated dienes (nmol/mg lipid) at 120 min.		
Components	Theoretical	Measured	SD (±)
Control	-	447.1	33.5
Lutein	-	177.3	36.1
Lycopene	-	208.2	79
Mixture	192.5	190	49.7

3.3 Lipophilic Antioxidants in Homogeneous Solutions. TEAC Assay

The additive antioxidant effect of carotenoids observed in a hydrophilic/lipophilic compartmented system, the unilamellar liposomes, was further investigated in a single comparment system (homogeneous solution), using the TEAC assay (see section 2.3.5). The carotenoids lutein, lycopene, zeaxanthin and α-carotene as well as α-tocopherol, were evaluated with respect to their antioxidant effect by measuring their antioxidant capacity in a homogeneous solution in ethanol as single compounds and in mixtures.

Single Compounds Four carotenoids including lutein, lycopene, zeaxanthin and β-carotene as well as α-tocopherol were measured in at least three different concentrations using the TEAC assay. All concentrations were tested in triplicate. The TEAC value of the measured compounds was calculated from the Trolox standard curve (see section 2.3.5). Figures 3.49 and 3.46 show the curves used to calculate the TEAC values of the measured compounds. Also displayed in the graph are the equations obtained from the linear regression curve fitted to the data points.

The results of the TEAC measurements point out that from the carotenoids tested the one exerting the most powerful antioxidant effect in a homogeneous environment is lycopene, with a Trolox equivalence of 2.56 and zeaxanthin being the weakest with 0.81. Table 3.12 presents the Trolox equivalents for the individual antioxidants; data are also graphically displayed (Fig. 3.44).

Table 3.12: Antioxidant TEAC values

Antioxidant	TEAC (μM Trolox Equivalents)
Trolox	1
Lutein	1.22 $\pm$ 0.11
Lycopene	2.56 $\pm$ 0.1
Zeaxanthin	0.81 $\pm$ 0.3
β-Carotene	1.88 $\pm$ 0.9
α-Tocopherol	0.92 $\pm$ 0.6

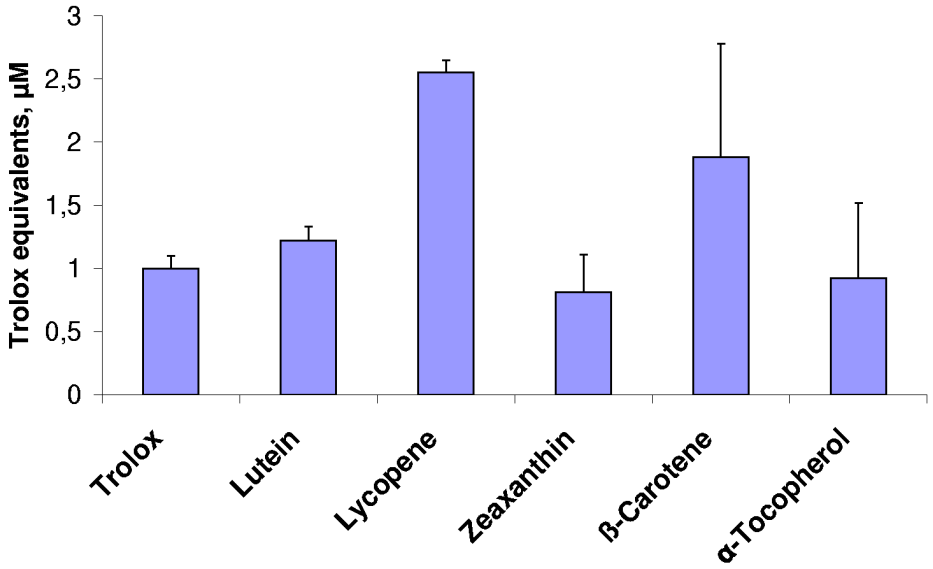

Figure 3.44: TEAC equivalents for the investigated antioxidants. The reference compound is Trolox which is fit to 1.

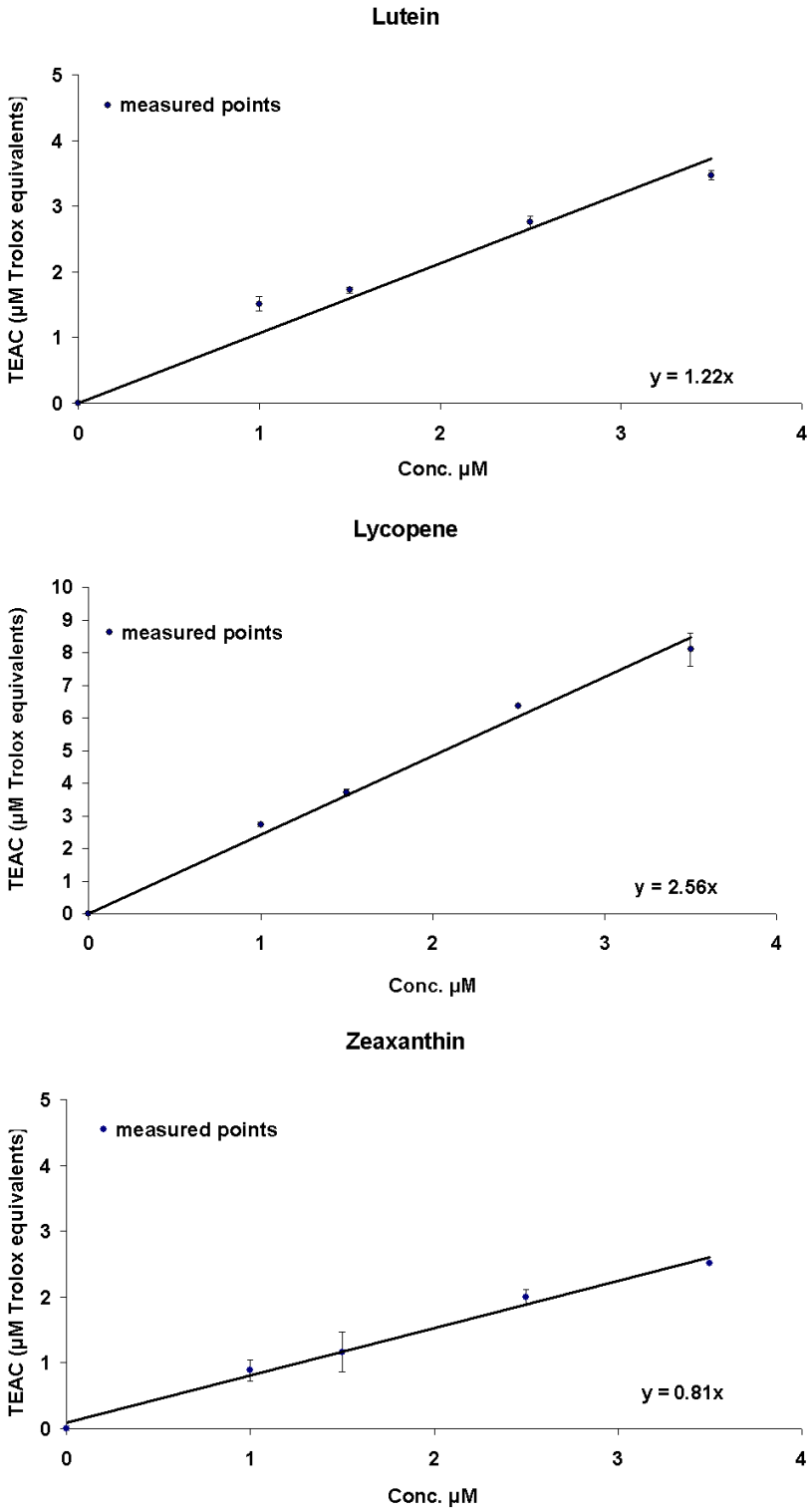

Figure 3.45: Linear regression curves fitted to the carotenoids data points in order to calculate the TEAC value.

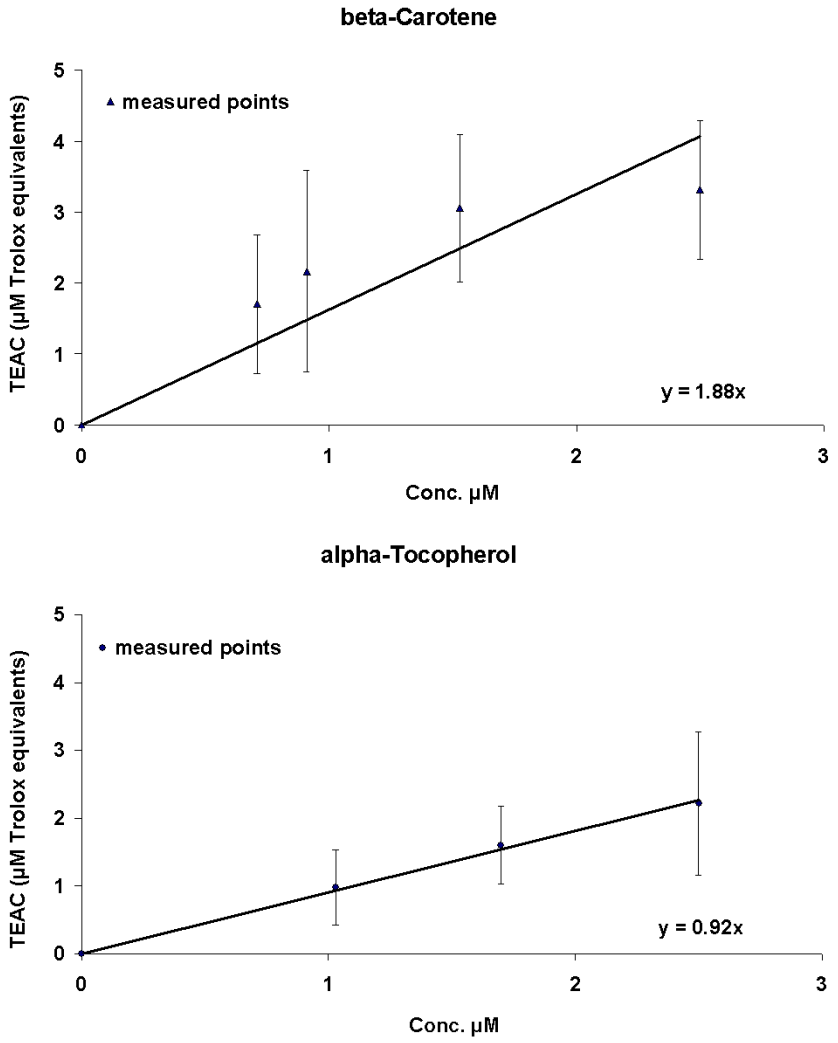

Figure 3.46: Linear regression curves fitted to β-carotene and α-tocopherol data points in order to calculate the TEAC value.

Mixtures of Lipophilic Antioxidants in a Homogeneous Environment After testing the lipophilic antioxidants as single compounds and determining their individual antioxidant capacity (TEAC values), combinations of these compounds were tested for possible synergistic antioxidant effects. Mixtures were investigated in at least triplicate. For every mixture, two different antioxidants were combined at different concentrations keeping one component at a constant concentration, the concentra-

tion of the second was gradually increased until an equimolar mixture was obtained. To confirm results, the inversed procedure is done by keeping the concentration of the second component constant while gradually increasing the concentration of the first one. For each of the final concentrations (at a certain combination of concentrations), a theoretical additive TEAC value was calculated. To obtain the theoretical additive TEAC value for a certain combination of concentrations, the TEAC values previously calculated for the single compounds are needed.

An example is shown for the mixture of lutein and lycopene, where lutein is kept at a constant concentration (3.5 μM) and lycopene´s concentration is gradually increased (from 0 to 3.5 μM). The theoretical additive TEAC value of the mixture of 3.5 μM lutein and 1.0 μM lycopene would then be 3.5 μM multiplied by lutein´s TEAC value of 1.22 (3.5 x 1.22 = 4.3) and added to 1.0 μM multiplied by lycopene´s TEAC value of 2.56 (1.0 x 2.56 = 2.56), that is (4.3 + 2.56) 6.86 TEAC units at a final concentration of 4.5 μM.

This way of calculating the theoretical TEAC value was applied to all combination of concentrations. The obtained **theoretical** additive values for the combination of concentrations were then compared with the **measured** TEAC values of the same combination of concentrations using the TEAC assay. If the value obtained from the TEAC measurements is higher than the calculated theoretical additive one, it can be assumed that the combination of the chosen antioxidants acts synergistically. If the measured TEAC values matches the calculated additive values, then the mixture acts additively. Are the measured TEAC values of the mixture lower than the calculated additive values, antagonistic effects may be operative.

Lutein and Lycopene Mixtures The mixture of lutein and lycopene was of special interest for two reasons, firstly their equimolar combination in unilamellar liposomes resulted in an additive effect. Secondly their levels where significantly increased in the intervention study after 12 weeks.

The following table (Table 3.13) shows TEAC values, theoretical and measured for carotenoids and mixtures. Also shown are the curves with the final concentrations of the carotenoid in mixtures plotted against their TEAC values (theoretical and measured).

Table 3.13: TEAC values from the combination of lutein and lycopene at different concentrations in a homogeneous environment. Calculated additive theoretical and measured TEAC values.

Mixture of lutein and lycopene

Concentrations μM			TEAC values	
Lutein	Lycopene	Mix. final	Theoretical	Measured ± SD (n=3)
3.5	0	3.5	-	3.5 ± 0.1
3.5	1.0	4.5	6.8	4.7 ± 0.1
3.5	1.5	5.0	8.1	5.7 ± 0.1
3.5	2.5	6.0	10.6	7.6 ± 0.02
3.5	3.5	7.0	13.2	7.7 ± 0.3
Lutein	Lycopene	Mix. final		
0	3.5	3.5	-	8.1 ± 0.52
1.0	3.5	4.5	10.1	7.4 ± 0.1
1.5	3.5	5.0	10.8	7.4 ± 0.2
2.5	3.5	6.0	12.0	7.6 ± 0.5
3.5	3.5	7.0	13.2	8.7 ± 0.2

Lutein was held at a constant concentration of 3.5 μM and mixed with different concentrations of lycopene ranging from 1.0 to 3.5 μM. The *measured* TEAC values of these mixtures were up to 40% lower than the *calculated* theoretical additive TEAC values for the same final concentrations of the mixtures. Inversing the combination of carotenoids at the same concentrations results in the same lower TEAC values, which can be taken from the curves where the final concentrations of these mixtures where plotted against their TEAC values (*theoretical* additive and *measured* TEAC values).

When lycopene was kept constant at 3.5 μM, it was observed that the *measured* TEAC values are lower than the calculated *theoretical* additive TEAC values. In both cases (lutein kept constant and lycopene gradually increased and vice-versa) lower TEAC values were measured than the calculated additive values for the same final concentration of this carotenoid mixture, indicating a less additive effect of

the combiantion of lutein and lycopene in a homogeneous solution.

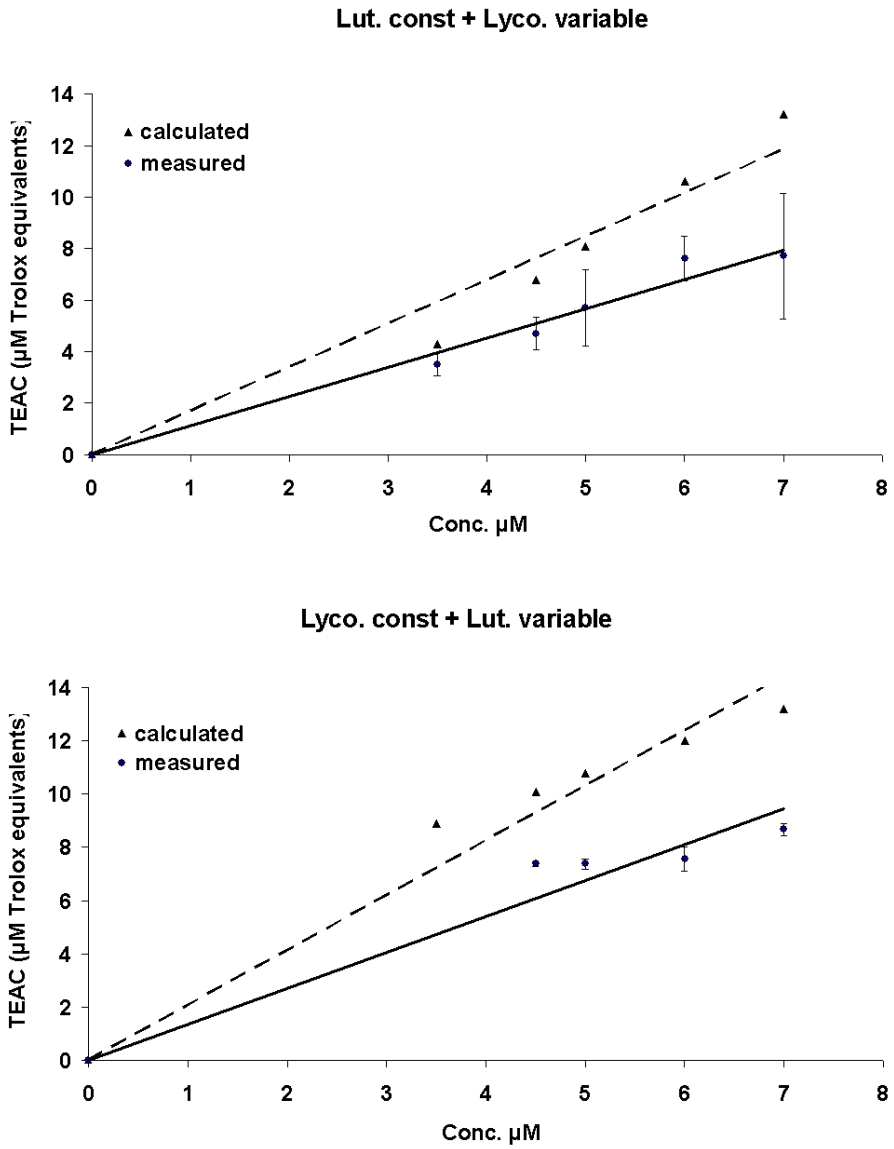

Figure 3.47: Calculated additive theoretical (dotted line) and measured (continuous line) TEAC values for mixtures of lutein and lycopene in a homogeneous solution. Upper panel: concentration of lutein kept constant at 3.5 μM and lycopene is gradually increased. Lower panel: concentration of lycopene kept constant at 3.5 μM and lutein is gradually increased.

Table 3.14: TEAC values from the combination of zeaxanthin and lutein at different concentrations in a homogeneous environment. Calculated additive theoretical and measured TEAC values.

Mixture of zeaxanthin and lutein

Concentrations μM			TEAC (μM TEAC values)	
Zeaxanthin	Lutein	Mix. final	Theoretical	Measured ± SD (n=3)
3.5	0	3.5	-	2.5 ± 0.11
3.5	1.0	4.5	4.0	4.0 ± 0.2
3.5	1.5	5.0	4.7	5.0 ± 1.0
3.5	2.5	6.0	5.9	5.1 ± 0.1
3.5	3.5	7.0	7.1	5.4 ± 0.3
Zeaxanthin	Lutein	Mix. final		
0	3.5	3.5	-	3.1 ± 0.10
1.0	3.5	4.5	5.1	4.0 ± 0.3
1.5	3.5	5.0	5.5	4.3 ± 0.1
2.5	3.5	6.0	6.3	5.1 ± 0.2
3.5	3.5	7.0	7.1	6.2 ± 0.05

Zeaxanthin and Lutein Mixtures The combination of these two carotenoids was chosen because both carotenoids are found in the *macula lutea* of the eye. The following table (Table 3.14) shows mixtures chosen to measure/calculate TEAC values. Also presented are the curves with the final concentrations of the carotenoid mixtures plotted against their TEAC values, theoretical and measured.

Zeaxanthin was held at a constant concentration of 3.5 μM and was mixed with lutein at concentrations ranging from 1.0 to 3.5 μM.

The calculated additive values were comparable to the measured TEAC values for the same final concentrations. Differences were observed at a final concentration of 6 μM (zeaxanthin at 3.5 μM and lutein at 2.5 μM). At this level the measured TEAC value (5.1) is lower than the calculated additive theoretical one (5.9). The mixture´s highest concentration of 7 μM, the difference is almost 2 TEAC units. The calculated theoretical additive value is 7.1 and the measured TEAC value for the same mixture is 5.4 TEAC units. When lutein is kept constant at 3.5 μM, and

zeaxanthin gradually increased from 1.0 μM to an equimolar mixture, the measured values are closer to the calculated additive values.

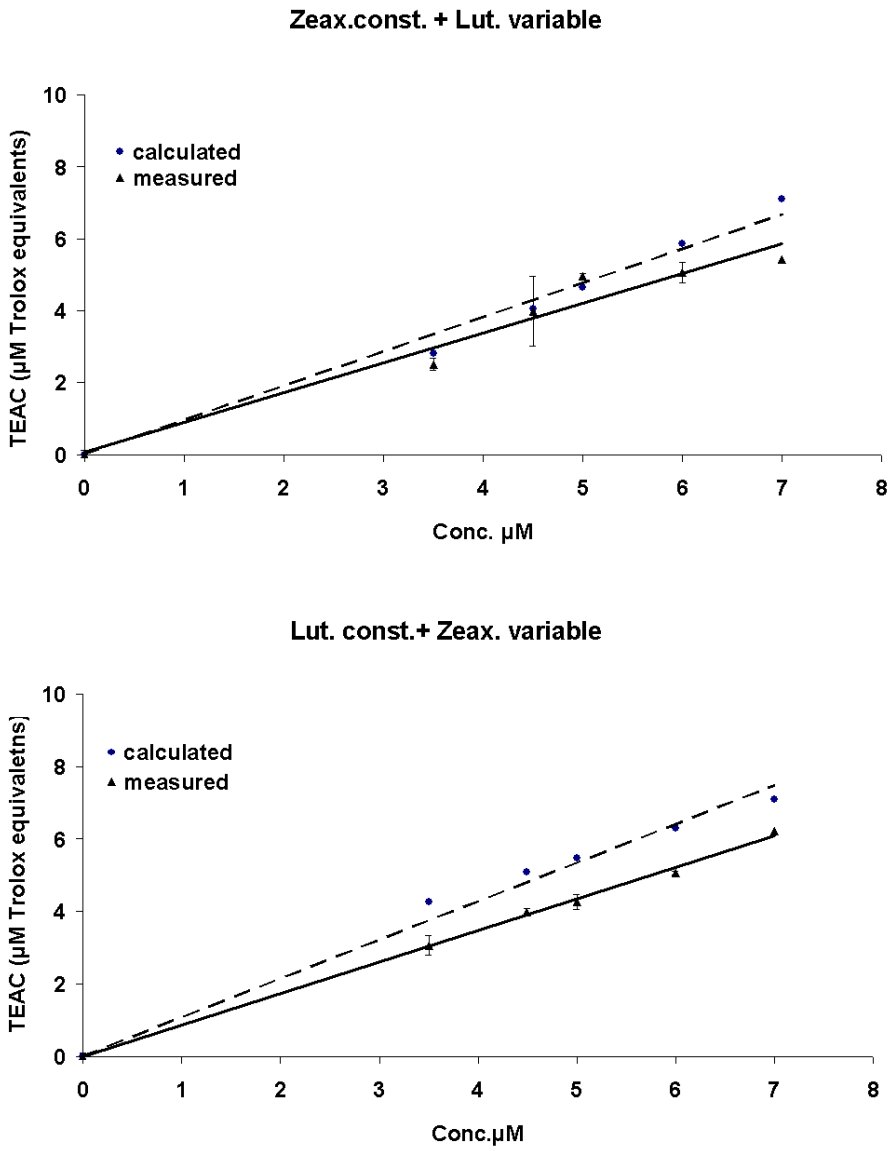

Figure 3.48: Calculated additive theoretical (dotted line) and measured (continuous line) TEAC values for mixtures of zeaxanthin and lutein in a homogeneous solution. Upper panel: concentration of zeaxanhtin kept constant at 3.5 μM and lutein is gradually increased. Lower panel: concentration of lutein kept constant at 3.5 μM and zeaxanthin is gradually increased.

Table 3.15: TEAC values from the combination of β-carotene and α-tocopherol in mixtures at different concentrations in a homogeneous environment.

Mixture of β-Carotene and α-Tocopherol

Concentrations μM			TEAC values	
β-Carotene	α-Tocopherol	Mix. final	Theoretical	Measured ± SD (n=3)
2.3	0	2.3	4.32	3.31 ± 1.0
2.3	0.71	3.01	4.98	5.5 ± 0.9
2.3	1.03	3.33	5.28	5.5 ± 1.2
2.3	1.7	4.0	5.9	6.9 ± 0.8
2.3	2.5	4.8	6.64	7.2 ± 1.0
β-Carotene	α-Tocopherol	Mix. final		
0	2.5	2.5	2.3	2.21 ± 1.05
0.7	2.5	3.2	3.63	3.0 ± 1.0
0.91	2.5	3.41	4.02	4.07 ± 0.7
1.53	2.5	4.03	5.19	5.47 ± 0.8
2.5	2.5	5.0	7.01	7.7 ± 1.2

β-Carotene and α-Tocopherol Mixtures β-Carotene is the predominant carotenoid in the human organism. It´s mixture with α-tocopherol was of special interest to investigate how a carotenoid may interact with vitamin E in homogeneous solutions. Table. 3.15 shows the results of the combination of β-carotene and α-tocopherol in a homogeneous solution.

The calculated additive TEAC values of the mixtures match the measured TEAC values, indicating that the mixtures of β-carotene and α-tocopherol have an additive antioxidant effect. β-Carotene was kept at 2.3 μM and mixed with α-tocopherol at concentrations ranging from 0.71 to 2.5 μM. An additive theoretical TEAC value was calculated for all mixtures, which were matched with measured TEAC value of the same mixtures.

Subsequently, α-Tocopherol was kept constant at 2.5 μM and mixed with β-carotene at concentrations ranging from 0.7 μM to 2.5 μM. An additive theoretical TEAC value was calculated for all mixtures and compared with measured TEAC

value of the same mixtures. Measured and calculated values match indicating that with inversed concentrations also an additive antioxidant effect is obtained.

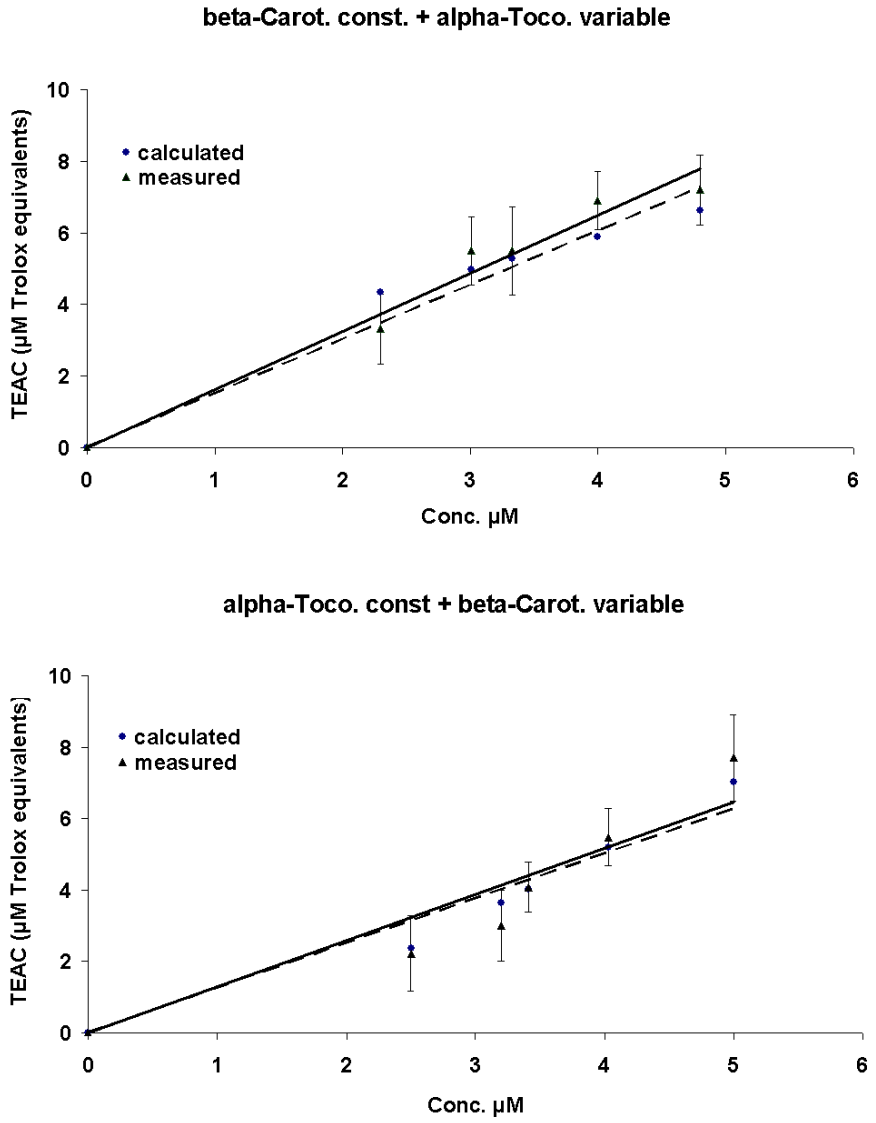

Figure 3.49: Calculated additive theoretical (dotted line) and measured (continuous line) TEAC values for mixtures of β-carotene and α-tocopherol in a homogeneous solution. Upper panel: concentration of β-carotene kept constant at 2.3 μM and α-tocopherol is gradually increased. Lower panel: concentration of α-tocopherol kept constant at 2.5 μM and β-carotene is gradually increased.

3.3.1 Summary Lipophilic Antioxidants in a Homogeneous Environment.

Lipophilic antioxidants including lutein, zeaxanthin, lycopene, β-carotene and α-tocopherol were measured individually and in selected mixtures in a homogeneous environment using the TEAC assay. In this environment the most efficient antioxidant was lycopene with a TEAC value of 2.56, the weakest effects were found for zeaxanthin with a TEAC value of 0.81 . None of the mixtures at the selected concentrations provided evidence for a synergistic antioxidant effect in this system. The selected combinations lead to additive effects. An equimolar mixture of lutein and lycopene was less active than expected from single compounds.

Chapter 4

Discussion and Conclusions

4.1 Discussion

Observational epidemiologic studies have shown that a high consumption of fruits and vegetables is associated with a decreased risk of chronic diseases and age related degenerative disorders. Currently, the recommendation is to increase the intake of a mix of fruits and vegetables. The world wide program "5 a Day" encourages people to take 5 portions or more of fruits and vegetables per day. The study presented was held within the concept of the "5 a Day" program in a healthy working population.

Study Population and Compliance Volunteers recruited for this study may be regarded as a well defined study population. The majority was female (96%), aged around early 50´s, part of a hospital staff, fairly or highly educated, following healthy habits: non-smoker majority (88%) and self-considered healthy (73%). Less educated persons or from socially disadvantaged backgrounds are less likely to follow diets rich in food and vegetables [67]. This socio-economical differences in fruit and vegetable intake are, however, cultural and regional dependent; Greek households of lower social class follow a healthier diet because of higher availability of vegetable oils, fresh vegetables, legumes, fish and seafood in this country [68]. In the U.K.

groups with lower social-economical backgrounds regard additional purchasing of fruits and vegetables (to reach the recommended 5 portions a day) as prohibitively expensive [69]. In Ireland although mean intake of vitamins and minerals was generally close to or above the recommended values, a positive relation was observed with healthy food intake and increased educational level [70]. Apart from the socio-economical background, sedentary life and gender seems to be an important factor related to higher consumption of fruits and vegetables [71].

The well defined population recruited for the present study was predominantly female and socio-economically well situated and non-sedentary. From the beginning of the study volunteers were highly motivated to follow a healthy diet or improving an already on-going habit of high intake of fruits and vegetables. This already on-going dietary habit taking high amounts of fruit and vegetable was surprising since 67% of the volunteers already had an optimal diet prior the start of the study. Fruit consumption was preferred over vegetables, a trend which persisted until the end of the study and was also reported elsewhere [72]. High intake in fruit and vegetable did not alter body mass index, which is in accordance with observations made in other trials [73] [72]. Despite the fact that the majority of the volunteers were following already a diet rich in fruit and vegetables, the intervention counseled diet did motivate the remaining participants, individuals with either a normal or a poor diet, to increase fruit and vegetable intake. Subjects with already high consumption of fruit and vegetables did move towards an even higher intake. After the 3 months of intervention compliance was almost total, clearly reflected by significant higher levels of blood antioxidant and the amount of fruit and vegetable consumed. It was also reflected by the migration pattern from a poor/normal nutritional state to an optimal one (see Figure 3.35 on page 103).

Markers of Exposure at Base Line Compared to other studies where plasma micronutrient levels were measured, volunteers in this study showed relatively high base line values of carotenoids and vitamins. This high base line values are well explained by the fact that almost two thirds of the participants where following a diet, though not necessarily of 5 portions of fruit and vegetables a day, but relatively high in fruits and vegetables. Table 4.1 shows micronutrient base line values of

different studies compiled from the literature.

The studies presented in Table 4.1 reflect data from free-living subjects with different backgrounds. It has to be noted that studies with free-living subjects are not easy to compare because subjects are usually recruited within a defined group resulting in small sample size and variation. In the case of a larger study population, information about volunteers background is not sufficient or often inaccurate due to self-reporting. For example in study B, the recruited men were staff members of a research institute (highly educated, higher socio-economical status), therefore not representative. Carotenoid base line levels of these male, smoking population are low compared to those observed in our study. Smoking habits seem to be an important factor influencing micronutrient levels [74], [76]. The smoking habit and gender of volunteers in study B could also explain the observed lower values. However, data from non-smoking female, healthy volunteers (study A) show lower levels of micronutrient compared to those observed in our study. Comparing the micronutrient base line levels of our study to those in a larger male/female population observed within a long period (study C, D) it can bee seen that though the range is similar, the levels found in our study population is above the average. Although levels of lutein in study C, D seem to be close to the levels observed in our study, study C and D lutein levels are given as sum of lutein and zeaxanthin. Vitamin C levels of our population was higher compared to other studies. Fat soluble vitamins, retinol and α-tocopherol were either lower or similar (base line) when compared to other studies. These observations are interesting, since they suggest that the observed high base line levels in carotenoids in the volunteers of our study represent a long-time exposure to these micronutrients due to high intake in fruit and vegetables, confirming the self-reported healthy lifestyle: in diet and mostly non-smoking.

Increased Levels in Markers of Exposure. Diet Response The intake of fruit and vegetables was assessed according to the profiles of micronutrients (carotenoids and vitamins) in blood plasma [20]. Micronutrient compounds in blood are collectively called *biomarkers of exposure*, since they confirm that an individual has been exposed to a diet rich in vitamins, carotenoids and/or other antioxidants. In this

Table 4.1: Plasma base line micronutrient levels in different study populations

	Studies				
	This study	A	B	C [a]	D
	n=112	n=64	n=21	n=136	n=1007
Parameter	males/females	females	males	males/females	females
	Germany	Germany	Netherlands	U.S.A	U.S.A
Lutein (μM)	0.37 ± 0.16	0.28 ± 0.12	0.15 ± 0.07	0.24 ± 0.12 [b]	0.32 ± 0.22 [b]
Zeaxanthin (μM)	0.08 ± 0.07	0.09 ± 0.04	0.05 ± 0.02	[b]	[b]
β-Cryptoxanthin (μM)	0.35 ± 0.36	0.25 ± 0.17	0.16 ± 0.08	0.14 ± 0.08	0.13 ± 0.11
Lycopene (μM)	0.45 ± 0.28	0.19 ± 0.15	0.41 ± 0.19	0.34 ± 0.18	0.44 ± 0.3
α-Carotene (μM)	0.12 ± 0.13	0.10 ± 0.13	0.05 ± 0.06	0.08 ± 0.08	0.07 ± 0.12
β-Carotene (μM)	0.65 ± 0.45	0.41 ± 0.31	0.30 ± 0.25	0.36 ± 0.33	0.3 ± 0.47
Retinol (μM)	1.35 ± 0.35	1.83 ± 0.58	2.20 ± 0.4	-	-
α-Tocopherol (μM)	28.9 ± 10.3	44.7 ± 12.3	26.2 ± 5.6	36.2 ± 13.8	23.22 ± 10.36
Vitamin C (μM)	55.1 ± 20.9	67.0 ± 19.2	41.0 ± 16.2	54.55 ± 26.14	36.93 ± 53.9
Vitamin B6 (nM)	48.3 ± 30.9	-	-	-	-

Note: values are means ± SD

[a] Plasma concentrations adjusted for BMI, alcohol intake, and serum cholesterol

[b] Sum of lutein and zeaxanthin levels

A = Healthy German women. Average age, 66 y [20].

B = Healthy Dutch men, smokers, aged 18-50 y [74].

C = Healthy men (34) and women (102), smokers, aged 18-35 y [75].

D = NHANES III 1988-1994 [76].

study high intake of fruits and vegetables (high exposure) resulted in higher levels of markers of exposure. After the intervention period, levels of lutein, lycopene α- and β-carotene were significantly increased compared to base line. β-Cryptoxanthin showed significantly lower levels and zeaxanthin levels remained at base line values. Significantly higher levels were observed for water soluble vitamins (vitamin C and vitamin B6) but not for lipid soluble vitamins (vitamin A and vitamin E). Levels of the latter remained at base line. The findings obtained from a descriptive statistical analysis were confirmed by reanalyzing the data adjusted for confounders. Our study showed that a 3 month intervention period is sufficient to increase levels of some carotenoids and vitamin C and vitamin B6, but it had no effect in elevating levels of retinol and α-tocopherol (vitamin A and vitamin E). Smith-Warner et al. reported a randomized, controlled dietary intervention study aiming to increase servings of fruit and vegetables during 12 months, in individuals (men and women) recently diagnosed with colorectal adenomatous polyps (at risk population) [72]. Individuals showed a significant increases in levels of carotenoids (lutein/zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene) and retinol, but interestingly not in lycopene nor in vitamin C despite the fact that vitamin C rich fruit consumption was significantly higher. α-Tocopherol and vitamin B6 were not measured. Furthermore, compared to base line, higher fruit and vegetable intake was achieved after six months of intervention, higher carotenoid levels after 3 months. This shows that our intervention program was very effective, since significant higher levels of some carotenoids were observed already after the first month of intervention were also 90% of the volunteers had achieved optimal fruit and vegetable intake. Comparing the fruit and vegetable intake in the study of Smith-Warner et al. [72] it is interesting to note that at base line, the intervened population had already a high intake of fruit and vegetables; about 8 servings/day. Volunteers in our study had similar base line levels in carotenoids with less servings. Even at the end of both studies, levels of some carotenoids were comparable although servings in the Smith-Warner study peaked at 13 servings/day. The way portions are defined can be misleading interpreting the results. However portions have been standardized and can be compared in both studies. Table 4.2 shows plasma levels of carotenoids at base line and at end of the intervention for our study and the one reported by

Table 4.2: Carotenoid levels in two intervention studies

	Studies			
	This study males/females (n=112)		Smith-Warner et al. [72] males/females (n=100)	
Parameter	Baseline	3 months	Baseline	12 months
Lutein (μM)	0.37 ± 0.16	0.41 ± 0.16*	0.34 ± 0.13 [a]	0.38 ± 0.14** [a]
Zeaxanthin (μM)	0.08 ± 0.07	0.08 ± 0.03	[a]	[a]
β-Cryptoxanthin (μM)	0.35 ± 0.36	0.27 ± 0.19*	0.14 ± 0.09	0.17 ± 0.09**
Lycopene (μM)	0.45 ± 0.28	0.51 ± 0.28*	0.72 ± 0.27	0.72 ± 0.25
α-Carotene (μM)	0.12 ± 0.13	0.18 ± 0.17*	0.12 ± 0.15	0.19 ± 0.16**
β-Carotene (μM)	0.65 ± 0.45	0.85 ± 0.62*	0.39 ± 0.42	0.40 ± 0.42**
Retinol (μM)	1.35 ± 0.35	1.33 ± 0.30	n.m.	n.m.
α-Tocopherol (μM)	28.9 ± 10.3	29.5 ± 9.80	n.m.	n.m.
Vitamin C (μM)	55.1 ± 20.9	63.6 ± 21.8*	n.a.	n.a.
Vitamin B6 (nM)	48.3 ± 30.9	64.4 ± 41.6*	n.m.	n.m.

Note: values are means ± SD
[a] Lutein and zeaxanthin levels are given together
n.a. = data presented in mg/day, not available in concentrations
n.m. = not measured
*p< 0.01
**p< 0.001

Smith-Warner et.al.

Levels in vitamin C can not be directly compared since in the study of Smith-Warner et al. [72] only daily intakes (mg/day) are given. However, by comparing carotenoid levels, subjects in our study, had higher carotenoid levels at base line levels and at study end (except lycopene). This can be explained by health conditions of the subjects recruited by Smith-Warner et al. [72]. Lower antioxidant profiles may be disease-related [20] despite very high intake of fruit and vegetables. These data suggest that healthy subjects need to consume less fruit and vegetables in order to reach comparable blood levels of antioxidants. Another explanation could may be related to food quality/type [77], [78] or ways of food preparation. Many of the vegetables consumed to meet the recommended 5 a day portions are not those that are most consistently associated with reduced disease risk, efforts to provide the benefits of fruits and vegetables in the diet can be controversial [79]. For instance, even though vegetable intake in the U.S. is 3.6 ± 2.3 portions/day,

only 25% of vegetables consumed are classified as being rich in putative protective phytochemicals which are associated with diminishing the risk of disease, e.g.: dark green and deep yellow vegetables and tomato products. In this scheme, increase in plasma α-carotene, β-carotene and lutein has been linked to the consumption of carrots and spinach. Lutein blood levels were particularly responsive to a diet where broccoli and cauliflower were ingested in increased amounts [77].

In the present thesis, the increase of a micronutrient in blood was correlated with the intake of a specific food type. Our findings are in accordance with the observation that lutein levels rise with consumption of spinach and broccoli [78], and in our study also with consumption cauliflower, cabbage and sprouts . Levels of α-carotene were correlated with the consumption of cooked vegetables but were negatively correlated with fruit juices. A clear correlation was found between high vitamin C blood levels and fruits like apple, pears and quince, which confirms the observation that in our study fruits were preferably consumed over vegetables [72]. However correlating levels of micronutrients to food types can give rise to confusing information (e.g. positive correlation of high lycopene levels and fish intake). Due to the structure and design of the food frequency questionnaire it proved to be useful tool to register and evaluate fruit and vegetable intake as well as compliance, but the questionnaire was of limited value in evaluating correlations of specific food intake and micronutrient levels.

Micronutrients and Biomarkers of Oxidative Stress Epidemiologically it has been shown that a diet rich in fruit and vegetables lowers the risk for degenerative diseases. Following the concept of biomarkers, the consumption of a functional food component is reflected by a marker of exposure. The exposure to a diet rich in those foods is confirmed by the levels of carotenoids and vitamins measured in blood. Furthermore, carotenoids and other micronutrients should have an effect on a markers of biological response. If markers of oxidative stress (MDA, protein carbonyls, homocysteine) respond to prooxidants, then it may be hypothesized that high intake in antioxidants should lower the levels of such markers of oxidative stress. Lowered levels of markers of biological response should be related to diminished damage to tissues (markers of intermediate endpoint) and may allow a prediction regarding a

long term effect on reducing the risk of disease. Although the counseled diet proved to be very effective in increasing carotenoid levels (which have antioxidant activity in vitro) as well as increasing levels of vitamin C and B6, no effect on levels of biomarkers of oxidative stress was observed.

The reasons for this unexpected findings are not clear. Some aspects are discussed in the following paragraphs.

Although carotenoids show clear antioxidant properties in vitro, data concerning an in vivo antioxidant effect of carotenoids are scarce. In an observational study, Anlasik et al. [80] reported that elderly subjects with a high fruit and vegetable intake had lower MDA and protein carbonyls levels compared to subjects with a low fruit and vegetable intake. MDA levels were inversely correlated with vitamin A and α-carotene, whereas protein carbonyls were inversely correlated with γ-tocopherol blood levels. This observations, however, do not explain causality. Lower protein carbonyl levels have been reported after 400g/day vitamin C dosing regimen on subjects with low basal antioxidant levels over one week [62]. However, dosing of specific antioxidants is not equal to dietary intervention, also duration and high dosage of supplementation may affect outcome [24], [25]. In another short-time intervention study on healthy females [81], a drink rich in antioxidants was given for a period of 2 weeks. Consumption of the juice did not alter blood or cellular antioxidant status or biomarkers of lipid peroxidation (MDA) or DNA damage (8-oxo-deoxyguanosine). It may be suggested that a further increase of dietary antioxidants in a healthy and already well-nourished population does not further improve health conditions and/or increase defense systems against oxidative damage.

Our study supports this view. Subjects in our study had already high levels of micronutrients acquired over a long time via the diet which were even further increased after the intervention. However, the healthy status of subjects, high base line levels of micronutrients, optimal intake of fruits and vegetables and significantly higher levels of micronutrients at the end of the study, did not affect levels of biomarkers of oxidative stress.

It can be proposed that constant high levels of antioxidants/micronutrients are related anyway to low levels of biomarkers of oxidative stress. However, this assumption is difficult to support since no reference values exist on biomarkers of oxidative

stress nor do recommended values for carotenoids exist, in plasma or tissues. A second possibility could be that the intervention period was too short to induce significant change in levels of biomarkers of oxidative stress. Although plasma levels of antioxidants may have reached a steady state in blood, deeper compartments may not have been saturated. The exchange of biomarkers between tissues and blood is not known.

The last point addresses the question if the biomarkers selected for the study are suitable to determine the situation of oxidative stress *in vivo*. Theoretically a higher availability of antioxidants (carotenoids and vitamin C) should shift a disbalance between antioxidants and pro-oxidants towards the side of antioxidants. This in turn would mean less damaged tissues and consequently lower levels of oxidative stress related products: MDA, protein carbonyls, 8-oxo-deoxyguanosine. So far most of the biomarker models rely on relationships found *in vitro*, or in animal models [52], [51], [82]. Clear human data relating biomarkers of exposure to those of oxidative damage and finally to clinical end point are scarce. The value of biomarkers has been challenged especially when used in human studies [83].

Micronutrients and Clinical Parameters A surprising result of the intervention study was that diagnostic parameters such as uric acid, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides were significantly higher at the end of the study. This trend was observed even after adjustment for confounders in the longitudinal analysis. Although significantly higher than base line, levels of these diagnostic parameters where within the acceptable and recommended range. Special attention was given to LDL-cholesterol, since high levels and oxidative modification of LDL play a role in the process of arterial wall lesion [84]. In a study by Neuhouser et al. [85], women with excellent or good diets (very high and high intake of fruits and vegetables) had significantly higher levels of palmitic acid which may rise serum concentrations of LDL [86]. This finding is also in agreement with a controlled feeding study [87] showing that when participants consumed a low-fat diet, there was a significant increase in palmitic acid incorporated into plasma phospholipids, compared to a high-fat diet. Although plasma phospholipid levels were not measured or characterized in our study, it is likely that despite there was

no restriction in fat intake, a high fruit and vegetable intake is associated with low-fat food consumption. Incorporation of palmitic acid into LDL is up-regulated by a low fat, high-carbohydrate diet. Although significantly higher (25% higher) than base line, LDL-cholesterol remained within recomended levels (93 mg/dL $<$ 160 mg/dL). Higher levels of circulating dietary antioxidants (e.g.carotenoids) might protect LDL against oxidation. In a study with low-fat, low-vegetable diet and low-fat, high-vegetable diet, it was surprisingly found that compared with the base line diet, the plasma concentration of oxidized LDL was equally elevated in both groups [84]. Plasma antioxidant levels were, however, increased only with the low-fat, high-vegetable diet.

Does restricting the diet to low-fat intake make sense?

Moderate intake of fat is advisable. One has to bear in mind that bioavailability of carotenoids (especially lycopene) is dependent on the co-ingestion of fat. The diet in our study was not low-fat restricting and this may explain the significant higher levels of triglycerides and lycopene levels observed.

A strong relation between high plasma homocysteine concentrations and CHD mortality has been reported [88], although the underlying biochemical mechanisms are not understood. Increased folate and vitamin B6 intake is reported to decrease homocysteine [89] [90]. In our study levels of vitamin B6 were significantly increased by high intake of fruit and vegetables but homocysteine levels were not affected. It may be necessary that in order to detect a direct effect of a rich fruit and vegetable diet on homocysteine levels, a more restrictive, well controlled diet on energy, fat, protein and carbohydrate has to be applied [91].

Model Systems. Lipsomes and TEAC Assay Model systems such as the TEAC assay are useful as a first-line approach evaluating unknown substances or substance mixtures for antioxidant effects. In the performed experiments with mixtures of carotenoids and α-tocopherol, an additive antioxidant effect of the selected compounds was observed. This observed effect was in accordance with the hypothesized value calculated for the measured mixtures. This suggest that at least in solution, the antioxidant effect of carotenoid mixtures can be theoretically predicted. This one-compartment model may be different when tested in a two-compartment model

such as unilamellar liposomes.

Stahl et al. [53] reported a synergistic effect of lutein and lycopene in multilamellar liposomes made by sonication. In the experiments with unilamellar liposomes carried out in this work, this effect could not be confirmed. In the equimolar mixtures of carotenoids tested in unilamellar liposomes only additive antioxidant effects were observed. Since carotenoids incorporated into liposomes may change membrane microviscosity, hydrophobicity, permeability to ions and diffusion of oxygen [92], [93] carotenoids in the structure of multilamellar liposomes made by sonication could probably decrease access of free radicals to lipids. In unilamellar liposomes, the lipid phase is in closer contact to the aqueous medium where the radical initiator AAPH was generated. The lack of improved protection against lipid peroxidation by carotenoids mixtures in our study is consistent with results reported elsewhere [94] and with recent results from intervention studies showing no effects of supplemented carotenoids on cardiovascular health [95].

4.2 Conclusions

The present work showed that a counseled diet within the framework of the world wide campaign of "5 a Day" was efficient in motivating individuals from a working population to increase their daily fruit and vegetable intake. The intervention study was not only effective in terms of increasing fruit and vegetable in individuals with an almost optimal intake of these foods but also in keeping participants motivation and interest as well as adherence to the program constant. Concomitantly to the increase in fruit and vegetable intake, levels of circulating antioxidants and micronutrients were significantly increased over base line.

Although levels of antioxidants and micronutrient were significantly increased by higher fruit and vegetable intake, biomarkers of oxidative stress were not affected by the intervention. Contrary to the hypothesized lowered values due to an increase in antioxidant/mirconutrient levels, biomarkers of oxidative stress remained at base line levels.

It may be suggested that a further increase of dietary antioxidants in a healthy and already well-nourished population as was the population enrolled in this study, does not further improve health conditions and/or increase defense systems against oxidative damage. Such an intervention program, which comprised education, information and monitoring of a diet high in fruit and vegetable intake might have an effect on markers of oxidative stress on a population with similar demographical characteristics but with lower micronutrient levels and lower fruit and vegetable intake.

A further consideration for the no-effect level of higher circulating antioxidant/micronutrient concentrations on markers of oxidative stress might have been the duration of the intervention study. Three months might have proved efficient in elevating levels of micronutrients, antioxidants and vitamins, but not long enough to exert an effect (at least not directly) on the chosen biomarkers of oxidative stress. These higher plasma levels of antioxidants may have reached a steady state in blood, however deeper compartments may not have been saturated. The exchange of biomarkers between tissues and blood is not known. Even higher fruit and

vegetable intake over already high-intake might require a longer period to observe an effect.

A final consideration is on the biomarkers chosen. The investigated markers of exposure (carotenoids, water and lipid soluble vitamins) were assumed to have a direct effect on the markers of oxidative stress. Epidemiologic studies have proved that a diet rich in micronutrients (e.g. carotenoids) is negatively correlated with the risk of oxidative stress related diseases. Carotenoids showing antioxidant effects *in vitro*, might however, have a different effect on biomarkers of oxidative stress (often free radical related), by other mechanisms than direct free-radical trapping.

In vivo modeling of antioxidant-pro oxidant interactions is difficult. Developing better *in vitro* systems to understand the *in vivo* situation is therefore required. Unilamellar liposomes are a good system to approach the investigation of such interactions and a better system than multilamellar liposomes. The bilayer membrane system can be constructed to mimic the structure of a cell membrane, which in this study delivered different results than those obtained with mutlilamellar liposomes using the same substances. Testing mixtures of lipophilic antioxidants in combination with water soluble antioxidants in the aqueous core of the liposome might be a powerful tool to understand the *in vivo* situation where antioxidant mixtures are present in different compartments of the cell rather than single, isolated compounds often tested with simple methods like the TEAC assay.

Summary

Epidemiological studies have shown a clear correlation between the increased consumption of antioxidant-rich foods and a decreased risk for several diseases related to oxidative stress. Consequently, a daily intake of at least 400 g of fruit and vegetables has been recommended by the WHO and the world wide campaign "5 a Day" aims at achieving this recommendation. In the frame of the present thesis an intervention study on a working population within the world wide campaign of "5 a Day" was carried out to test whether a counseled diet of five portions of fruits and vegetables a day for a period of 3 months, leads to an increase in antioxidant/micronutrient levels in blood and consequently to a decrease in biomarkers for oxidative stress and cardiovascular diseases. Blood levels of antioxidants/micronutrients as well as biomarkers for oxidative stress and cardiovascular diseases were analyzed by means of HPLC and ELISA. Another aspect of the study was to evaluate volunteers' compliance and collect information about the consumption of specific food items. Increases in micronutrient levels (especially carotenoids) were compared and correlated to intake frequencies of selected food.

The present work shows dietary counseling is effective in motivating individuals from a working population to increase their daily fruit and vegetable intake. The intervention not only increased fruit and vegetable intake of individuals with an almost optimal consumption of these foods, but also kept participants motivated and interested. Concomitantly to the increase in fruit and vegetable intake, levels of circulating antioxidants and micronutrients were significantly increased over base line. Although levels of antioxidants and micronutrient increased, biomarkers of oxidative damage were not affected by the intervention.

It is concluded that a further increase of dietary antioxidants in a healthy and already well-nourished population has no lowering effect on the selected biomarkers of diseases related to oxidative stress. No apparent improvement of health conditions and/or benefit from an increased defense systems against oxidative damage were observed. The lack of an effect on biomarkers of oxidative stress may be due to the short duration of the intervention (3 months). Also the suitability of the biomarkers as selective indicators for oxidative stress conditions or cardiovascular diseases may be questionable.

Additionally, those carotenoids, which increased in blood levels, were further tested for their antioxidant properties alone and in combinations to determine possible synergistic effects in liposomes. Carotenoid mixtures and single compounds were also evaluated for antioxidant potency and synergism in a single compartment model using homogeneous solutions. In vitro systems provide a suitable tool to understand the antioxidant properties of dietary constituents. It has been shown that a bilayer membrane system such as unilamellar liposomes, can be used to mimic the structure of a cell membrane to test antioxidants for additive and/or synergistic effects. In the present work mixtures of carotenoids in unilamellar liposmes had an additive antioxidant effect in oxidative stress conditions. Furthermore the model of unilamellar liposomes is superior to that of multilamellar systems when investigating antioxidant effects in vitro.

Combining results from in vivo modeling of prooxidant/antioxidant reactions with intervention studies is essential to understand mechanisms of oxidative damage and and develop strategies of defense including the use of dietary components.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Die Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt und auch noch nicht veröffentlicht.

Düsseldorf, April 2006 Juan-Carlos Carrillo Peláez