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*Chapter XIV*

# Biology of Carotenoids and their Potential Cardiovascular Health Benefits

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## Abstract

More than 700 carotenoids have been identified, but a few of them are considered of nutritional relevance and circulate at micromolar levels, therefore they receive the most attention from health researchers. Among them, β-carotene, α-carotene and β-cryptoxanthin are the major carotenoids having significant pro-vitamin A activity, while lutein, lycopene, and zeaxanthin are not converted into active retinoids by humans. Their biological activity is therefore independent of retinoid-associated pathways. Given their chemical structure, carotenoids have been conjectured to act as free-radical scavengers, even though they can act as pro-oxidant molecules, at least at high oxygen concentration. However, more recently, several biologically beneficial activities relating to their ability to regulate various cellular functions have been proposed. Of note, a number of epidemiological studies have shown a correlation between elevated dietary carotenoid intake and circulating levels and decreased risk of cardiovascular disease (CVD). It has recently been demonstrated that circulating serum carotenoids are associated, beneficially it would seem, with markers of inflammation, oxidative stress, and endothelial dysfunction, which are known to be associated with CVD.

At present, it is widely accepted that one of the earliest detectable pathogenic events in both human and experimental atherosclerosis is vascular inflammation associated with activation of NF-κB pathway, in turn triggering up-regulation of the expression of the vascular cell adhesion molecules (VCAM-1), intercellular cell adhesion molecules (ICAM-1) and E-Selectin.

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Nitric Oxide (NO), constitutively generated by the endothelial cells, plays an important role in maintaining vascular homeostasis and the pro-inflammatory response that characterizes the early stages of atherosclerosis. It is known that NO inhibits the vascular inflammatory response, down-regulating NF-κB-dependent expression of adhesion molecules. The maintenance of endothelial NO bioavailability is therefore considered beneficial to endothelial functions and more in general to vascular health. However, in the dysfunctional endothelium, NO may rapidly react with superoxide anion ($O_2^-$) to form a stable potent oxidant peroxynitrite ($ONOO^-$) resulting in decreased vascular relaxation, and contributing to the up-regulation of NF-κB dependent cellular response. Thus, the general effect of anti-oxidant molecules on the biological function of NO is likely to be due, at least in part, to a direct removal of $O_2^-$. Within this scenario, carotenoids may be considered potential anti-oxidant modulators of endothelial response to pro-oxidant/inflammatory stimuli.

Even though *in vitro* and *in vivo* experiments have recently demonstrated that carotenoids are able to reduce inflammation, while epidemiological studies indicate a strong correlation between dietary carotenoid consumption and decreased risk of CVD, the mechanism underlying the carotenoid's cardiovascular protective activities, is still little known.

**Keywords**: Carotenoids, Endothelial dysfunction, Inflammation, Nitric Oxide, Reactive Oxygen Species, Cardiovascular health

# Introduction

Carotenoids are tetraterpenoids that are synthesized by plants and microorganism but not by animals. Various fruits and vegetables form the main source of carotenoids in the human diet [1, 2]. They are a class of pigmented compounds which have long been mooted as cardiovascular disease-preventive food constituents.

More than 700 carotenoid compounds have been characterized; about 50 of them consumed in the human diet [3, 4]. About 10-15 carotenoids represent most of the dietary intake, and these are found in measurable concentrations in human blood and tissues [5, 6] the most common being lycopene, lutein, β-carotene, α-carotene, β-cryptoxanthin, and zeaxanthin [7].

Various biological effects have been ascribed to carotenoids. One possible action mechanism of carotenoids is via their antioxidant and scavenging capacity [8]. There is, in fact, reason to think that carotenoids act as modulators of intracellular redox status [9]. The conjugated double bond structure is primarily responsible for the chemical reactivity of carotenoids with free radicals such as the peroxyl, hydroxyl, and superoxide radicals. Of note, carotenoids have proved able to prevent or decrease oxidative damage to DNA, lipid and proteins [3, 10].

Conversely, a number of reports claim that carotenoids can act as pro-oxidant molecules and increasing the total radical yield in a system [11, 12]. The key factor to determine the switch of carotenoids from antioxidant to pro-oxidant is the oxygen partial pressure (pO2) and the carotenoid concentration [13, 14]. At higher pO2 a carotenoid radical can react with molecular oxygen to generate a carotenoid-peroxyl radical which can act as a pro-oxidant by promoting oxidation of unsaturated lipids.

Although the antioxidant properties of carotenoids have been proposed as the main mechanism behind their beneficial effects, recent studies are also beginning to show that these compounds may mediate their effects via other mechanisms such as cell growth relulation, gap-junction communication, modulating gene expression, etc [7]. In addition, carotenoids are a sizable dietary source of vitamin A, dividing into provitamin A (β-carotene, α-carotene and β-cryptoxanthin) and non-provitamin A compounds [15].

Interestingly, a number of epidemiological reports have shown a correlation between elevated dietary carotenoid intake and circulating levels and prevention of CVD [16, 17]. For example a relationship has recently been demonstrated between circulating carotenoid concentrations and several markers of inflammation, oxidative stress, and endothelial dysfunction [18], which are known to be associated with CVD [19-21].

At present it is well recognized that atherosclerosis is an inflammatory disease [22], and there is some evidence to suggest that the beneficial effects of carotenoids may result from modulation of the inflammatory responses.

NF-κB inflammatory pathway activation, in turn triggering up-regulation of the expression of the VCAM-1, ICAM-1 and E-Selectin [23], has been shown to be partially regulated by Radical Oxygen Species (ROS) and has been implicated in various forms of CVD [24, 25]. Moreover, NO, constitutively generated by endothelial cells, plays an important role in the maintenance of vascular homeostasis and in the pro-inflammatory response that characterizes the early stages of atherosclerosis [26]. An imbalance entailing reduced production of NO and increased production of ROS may be involved in impaired endothelium-dependent vasodilation in patients with cardiovascular risk factors and diseases. It is known, in fact, that NO inhibits the vascular inflammatory response by quenching NF-κB nuclear transfer thanks to its regulatory activity on IκBα synthesis [27] and also by directly inhibiting NF-κB binding to DNA [28]. These events, in turn, down-regulate NF-κB-dependent expression of adhesion molecules [29].

Carotenoids and vitamins could have an antioxidant-mediated tempering influence on endothelial function and inflammation, thereby reducing the risk of atherosclerosis [30]. We recently demonstrated that β-Carotene or lycopene treatment reduce the inflammatory response in tumor necrosis factor-α (TNF-α)-treated human umbilical vein endothelial cells (HUVECs). This is due to redox balance protection and to the maintenance of NO bioavailability [31]. Lee and colleagues demonstrated that Akt-specific inhibitor reverses the inhibitory effect of carotenoids on tissue factor activity, indicating that carotenoids enhance phosphorylation of Akt and suppress tissue factor activity in endothelial cells potentially through a NO-mediated mechanism [32]. This would support the idea that maintenance of endothelial NO bioavailability may be beneficial to endothelial function and more in general to vascular health. In line withthis hypothesis, an astaxanthin-enriched diet reduces endothelium-mediated blood pressure and improves cardiovascular parameters in *in vivo* experiments performed on spontaneously hypertensive rats [33]. In a human study, George and colleagues [34] recently demonstrated significant effects of chronic and acute consumption of fruit- and vegetable-puree-based drinks on endothelium-mediated vasodilation, known to be due to regular NO availability.

However, although the studies mentioned support the idea that carotenoids may exert their cardiovascular protective action by increasing NO bioavailability, the majority of papers published to date indicate that such benefits stem from carotenoids' anti-inflammatory action, though the mechanism/s underlying this vascular activity is/are still largely unknown.

Martin et al. [35], showed that preincubation of lycopene using human aortic endothelial cell cultures resulted in a 13% decreased expression of the vascular cell adhesion molecule. Further study confirmed that lycopene also inhibited LPS-induced IκB phosphorylation, IκB degradation, and NF-κB translocation. Moreover, lycopene blocked the phosphorylation of ERK1/2 and p38 MAP kinase but not c-Jun NH2-terminal kinase [36].

Notably, Riso et al. [37] have reported that concentrations of the proinflammatory cytokine TNF-α in the blood of healthy volunteers were decreased after dietary supplementation with a tomato-based drink.

However, because carotenoids are a complex group of chemicals, and studies of the health effects of carotenoids are very heterogeneous, it is difficult to perform a meta-analysis or even a detailed systematic review of the health effects of carotenoids.

Thus, although plasma concentrations of carotenoids are considered useful biomarkers of total dietary intake of vegetables and fruit [38] while epidemiological studies have provided convincing evidence in support of the protective role of carotenoids in CVD [39], these observations need to be validated on the one hand by carrying out *in vitro* studies on the molecular mechanisms and, on the other hand by conducting well controlled human intervention studies in the future.

The present chapter will outline the current situation of relations between the carotenoids and prevention of CVD, examining epidemiological studies, clinical trials and *in vitro* experiments as well as *in vivo* animal studies. As a final point, a conclusion as to the importance of these compounds in cardiovascular health will be drawn.

## Epidemiological Studies

In the scientific research community there has been keen interest in the relationship between diet and health outcomes. Numerous epidemiological studies over the past decades have shown that protection against many chronic diseases, including CVD, is due to high consumption of fruit and vegetables. Recently, Donaldson analyzed sixty-two studies of plasma carotenoids in relation to health outcomes, mostly prospective cohort studies or population-based case-control studies. Based on these data, a carotenoid health index has been proposed with risk categories as follows: very high risk: <1 μM, high risk: 1–1.5 μM, moderate risk: 1.5–2.5 μM, low risk: 2.5–4 μM, and very low risk: >4 μM [40]. Notably, over 95 percent of the USA population falls into the moderate or high risk category of the carotenoid health index [41].

Today, CVD is the primary cause of death in Western cultures and accounts for up to a third of all deaths worldwide. In comparison to the Northern European or other Western countries, the Mediterranean area has lower rates of mortality from cardiovascular diseases and cancer, and this is ascribed, at least in part, to the so-called Mediterranean diet, which is rich in plant-derived bioactive phytochemicals [42]. Recognition of the active constituents of the Mediterranean diet is therefore fundamental to any formulation of correct dietary guidelines. Several articles have appeared in support of the role of carotenoids in the prevention of CVD, mostly based on epidemiological studies showing a relationship between carotenoid plasma levels and CVD. A less well-defined and more multifaceted picture emerges from interventional trials, where a number of works have reported contradictory

results (see below). Although many features of carotenoids' *in vivo* metabolism, functions and clinical indications remain to be clarified, supplementation of low doses of these compounds has been already suggested as a preventive measure for combating and improving many aspects of CVD.

In recent times, 139 Cretan (Greek) men aged 79 years and over were compared to men from Zutphen (The Netherlands). The Cretan men had approximately fourfold higher mean levels of lycopene as well as a lower level of oxidative stress and higher levels of antioxidants in plasma than men of the same age from Zutphen [43].

Lately, Karppi et al. [44] assessed relations between the concentrations of serum carotenoids and CVD mortality among Eastern Finnish men, demonstrating that low concentrations of serum β-carotene concentrations may increase the risk for CVD mortality among Eastern Finnish men. In addition, the same authors had previously evaluated serum samples from 349 subjects in relation to concentrations of conjugated dienes in low-density lipoprotein (LDL), this being one marker of lipid oxidation. The lycopene content in plasma correlated significantly and negatively with the content of conjugated dienes. Thus, dietary carotenoids proved significantly to lower LDL oxidative modification *in vivo* [45]. To examine whether serum concentrations of carotenoids are related to the risk of sudden cardiac death (SCD) in middle-aged men, Karppi et al. [46] studied a population consisting of 1031 Finnish men aged 46-65 years enllisted in the Kuopio Ischemic Heart Disease Risk Factor (KIHD) cohort. Their results suggest that low serum β-carotene concentrations may enhance the risk of SCD in middle-aged Finnish men. Additionally, low serum-carotene concentrations may be associated with the risk of CVD and total mortality. Early observational studies reported an association between a high dietary intake of β-carotene and reduced incidence of CVD [47, 48]. In a case-control study, the risk of nonfatal acute myocardial infarction (MI) in women was inversely associated with daily intake of $\beta$-carotene-containing diet [49]. In the Rotterdam study, a population-based cohort study targeting the elderly, the dietary intake of $\beta$-carotene was inversely associated with the risk of MI [50].

Interestingly, in the American Health Professional's Study conducted on 39,910 US males, the carotene intake was associated with a lower risk of Coronary heart disease (CHD) among current smokers but not nonsmokers [51].

To search for associations between serum carotenoids and risk factors for development of atherosclerosis, Xu et al. [52] studied 40 early atherosclerosis patients without clinical cardiovascular events, and comparable healthy controls. The results suggested that early atherosclerosis patients had lower serum concentrations of lutein and zeaxanthin than healthy subjects. Serum carotenoids were associated with reduced risk of atherosclerosis. This association was further supported by the study of Dwyer et al. [53] on 573 middle-aged women and men who were free of symptomatic cardiovascular disease at baseline. The findings suggest that higher levels of plasma oxygenated carotenoids (lutein, zeaxanthin, beta-cryptoxanthin) and α-carotene may be defensive against early atherosclerosis. Supporting this observation, Marin et al. [54] demonstrated that the Mediterranean diet induces a decrease in endothelial injury and dysfunction, which is associated with an improvement in the regenerative capacity of the endothelium in elderly subjects. Notably, recent findings show that the favourable effects of fruit and vegetable intake on markers of inflammation and oxidative stress are already present by early puberty [55]. In addition, Azzini et al. [56] showed that the Mediterranean dietary pattern is associated with significant

amelioration of multiple risk factors, including a better cardiovascular risk profile, reduced oxidative stress and modulation of inflammation.

However, evidence regarding the health benefits of carotenoids is controversial. In recent times, the effects of serum carotenoids and their interactions on mortality have been examined in a representative sample of US adults. The study consisted of adults aged ≥20 years enrolled in the National Health and Nutrition Examination Survey (NHANES) III, 1988–1994, with measured serum carotenoids and mortality (CVD and cancer) follow-up through 2006 (N=13,293). Analyses with continuous carotenoids confirmed associations of serum total carotenoids, α-carotene, and lycopene with all-cause mortality ($P$<0.001). In a random survival forest analysis, very low lycopene was the carotenoid most strongly predictive of all-cause mortality, followed by very low total carotenoids. α-carotene/β-cryptoxanthin, α-carotene/lutein+zeaxanthin and lycopene/lutein+zeaxanthin interactions were significantly related to all-cause mortality ($P$<0.05). Interestingly, low α-carotene was the only carotenoid associated with CVD mortality ($P$=0.002). Very low serum total carotenoid, α-carotene, and lycopene concentrations may be risk factors for mortality, but carotenoids show interaction effects on mortality. Studies of balanced carotenoid combinations are necessary for confirmation [57].

Moreover, several years ago the EURAMIC study already suggested that lycopene, or some highly correlated substance which is found in a common food source, may contribute to the protective effect of vegetable consumption on myocardial infarction risk [16]. Subsequently, De Waart et al. [58] suggested that serum levels of individual carotenoids, particularly the oxygenated species, are inversely associated with all-cause mortality.

Recently, epidemiological data concerning lycopene and its potential cardiovascular health benefits have been extensively reviewed by Bohm [59]; the fundamental studies are summarized in this chapter and shown below.

A total of 264 serum samples obtained from healthy Korean women were analysed for their lycopene content [60]. In addition, arterial stiffness - a possible marker involved in the pathophysiology of CVD - was assessed by brachial-ankle pulse wave velocity (baPWV). Serum lipid profile, high-sensitivity C-reactive Protein (hs-CRP) and contents of oxidized low density lipoprotein (oxLDL) were also analysed. A negative correlation was found between lycopene and oxLDL and also between lycopene and baPWV. Thus, lycopene may be responsible for a reduced oxidative modification of LDL, this possibly being one mechanism by which lycopene could reduce arterial stiffness and the risk of CVD.

Again, a total of 299 Korean men were investigated as to the interrelationship between arterial stiffness, antioxidant status and the risk of metabolic syndrome. The authors analysed, among other parameters, baPWV, lycopene content, lipid profile and oxLDL. baPWV inversely correlated with lycopene content in serum. A negative correlation was also seen between lycopene and oxLDL. Thus, an interrelationship was shown between circulating lycopene, baPWV and metabolic syndrome [61].

In another study, 3061 participants were invited to fill in a questionnaire and to give serum samples. Lycopene contents in serum tended to be lower for those who died due to CVD than for those who survived [62]. A case-control study with 760 cases and 682 controls showed a decreased risk of acute myocardial infarction with increasing intake of α-carotene, β-carotene and β-cryptoxanthin but no association for lycopene [63]. The CARDIA Study (Coronary Artery Risk Development in Young Adults) with 4580 participants showed that

those people with higher lycopene contents tended to have less healthy lifestyles. Serum total and individual carotenoids, with the exception of lycopene, were inversely associated with markers of inflammation, oxidative stress and endothelial dysfunction [18]. The Minnesota Heart Survey Study with 5369 men and 6070 women used a 24-h dietary recall. The authors developed a Heart Disease Prevention Index. This index improved between 1980/1982 and 2000/2002: for men by 2.58 points (8.3%) from 31.14 to 33.72 and for women by 2.44 points (7.9%) from 30.97 to 33.41. Thus, overall diet quality has moderately improved. However, improvement plateaued and levelled off during the last 5-year period that was studied. Regarding the carotenoids, only uptake of β-carotene, lutein, zeaxanthin and β-cryptoxanthin significantly increased from 1980/1982 to 2000/2002, but not consumption of lycopene [64]. So, controversial epidemiological data exist regarding the CVD-preventive effects of lycopene.

## *In Vitro* Experiments

As mentioned above, carotenoid-rich diets have been associated with decreased risk of CVD, but the underlying mechanism is still unknown.

In the last ten years some *in vitro* studies have suggested that carotenoids significantly inhibit TNF-α-induced ICAM-1 and VCAM-1 expression in both vein and arterial endothelial cells [65] and have barrier integrity activity, as well as inhibitory activity on cell adhesion and migration to endothelium by blocking the activation of NF-κB, CD14 and TLR4 expression and production of TNF-α [66].

We recently demonstrated that in HUVECs, both β-carotene and lycopene significantly affected TNF-α-induced inflammation. Notably, this was associated with a significant decrease in the generation of ROS and nitrotyrosine (an index of $ONOO^-$), increased NO/cGMP (cyclic guanosine monophosphate) levels, and down-regulation of NF-kB-dependent adhesion molecule expression and monocyte–HUVEC interaction. Thus, our results indicate for the first time that treatment with β-carotene or lycopene reduces the inflammatory response in TNF-α-treated HUVECs through redox balance protection and the maintenance of NO bioavailability [31].

The maintenance of endothelial NO bioavailability is therefore considered beneficial to endothelial function and more in general to vascular health. However, in TNF-α-stimulated endothelium NO rapidly reacts with superoxide anion ($O_2^-$) to form a stable potent oxidant $ONOO^-$ resulting in decreased vascular relaxation, and contributing to the up-regulation of NF-kB-dependent cellular response. Thus, the general effect of anti-oxidant molecules on the biological function of NO is likely to be due, at least in part, to direct removal of $O_2^-$ [67]. Within this scenario, carotenoids may be considered potential anti-oxidant modulators of endothelial response to pro-oxidant/inflammatory stimuli.

Again, the effects of lycopene on oxidative injury and apoptosis in endothelial cells following exposure to $H_2O_2$ were investigated by Tang et al. [68] using human vascular endothelial cells (ECV304 cells). Pre-treatment with lycopene dose-dependently decreased malondialdehyde (MDA) contents in $H_2O_2$-treated cells. Lycopene also significantly reduced the number of cells undergoing apoptosis in response to H2O2 inhibiting the upregulation of p53 messenger ribonucleic acid (mRNA) and caspase3 mRNA. These results tend to suggest

that protecting endothelial cells from oxidative injury may be one of the mechanisms underlying the cardiovascular-related beneficial effects of lycopene. In agreement with the hypothesis that carotenoids might exert their protective role through oxidative stress reduction, Rossoni-Junior et al. [69] recently showed that neutrophils from diabetic animals produce significantly more reactive oxygen species and NO than their respective controls and that supplementation with beta carotene and annatto (which has been identified as a carotenoid having antioxidative effects) is able to modulate the production of these species. Interestingly, annatto extract may have therapeutic potential for modulation of the reactive oxygen species/NO balance induced by diabetes. The study published by Bai et al. [70] in 2005 already supported the idea that carotenoids may protect against oxidative stress. β-carotene, in fact, directly blocked the intracellular accumulation of reactive oxygen species in RAW264.7 cells stimulated with LPS, just as both the NADPH oxidase inhibitor diphenylene iodonium and antioxidant pyrrolidine dithiocarbamate did. The inhibition of NADPH oxidase also inhibited NO production, inducible Nitric Oxide (iNOS) expression, and iNOS promoter activity. Moreover, carotene inhibited the expression and production of these inflammatory mediators in both LPS stimulated RAW264.7 cells and primary macrophages in a dose-dependent fashion, as well as in LPS-administrated mice. Furthermore, this compound suppressed NF-κB activation and iNOS promoter activity in RAW264.7 cells stimulated with LPS. These results suggest that β-carotene possesses anti-inflammatory activity by functioning as a potential inhibitor of redox-based NF-κB activation, probably due to its antioxidant activity.

Astaxanthin, a xanthophyll carotenoid, is a nutrient with unique cell membrane actions. This molecule neutralizes free radicals or other oxidants by either accepting or donating electrons, and without being destroyed or becoming a pro-oxidant in the process. Its linear, polar-nonpolar-polar molecular layout equips it to precisely insert itself into the membrane and span its entire width.

In this position, astaxanthin can intercept reactive molecular species within the membrane's hydrophobic interior and along its hydrophilic boundaries. In cultured cells, astaxanthin protected the mitochondria against endogenous oxygen radicals, conserved their redox (antioxidant) capacity, and enhanced their energy production efficiency. The concentrations used in these cells would be attainable in humans by modest dietary intakes [71].

Thus, increasing evidence suggests that carotenoids may protect against atherosclerosis, although, the exact mechanism(s) is still unknown. Because carotenoids may be considered efficient antioxidants, it has long been proposed that this property may be responsible for its beneficial effects. However, recently other mechanisms such as modulation of lipid metabolism through control of cholesterol synthesis and oxysterol toxic activities have been evoked as relevant effects [72].

Consistent with this hypothesis, Palozza et al. recently demonstrated that lycopene (0.5–2 mM) dose-dependently reduced the intracellular content of total cholesterol in THP-1 cells. This effect was due to a reduction in expression of 3-hydroxy- 3-methylglutaryl-coenzym-A (HMG-CoA) reductase, an enzyme promoting the deacylation of HMG-CoA to mevalonate [73]. As hypercholesterolaemia is one of the most important risk factors for atherosclerosis, these results imply a potential role of lycopene in attenuating foam cell formation and thus in CVD risk reduction. Lycopene (0.5–2 mM) also dose-dependently reduced 7-ketocholesterol-induced ROS production and 8-hydroxydeoxyguanosine formation in human THP-1

macrophages. In addition, lycopene was able to counteract 7-ketocholesterol-induced apoptosis by limiting caspase-3 activation [74]. Moreover, in the same cellular model lycopene prevented oxysterol-induced increase in pro-inflammatory cytokine secretion and expression. That effect was accompanied by inhibition of oxysterol-induced ROS production, mitogen-activated protein kinase phosphorylation and NF-κB activation. In additon, the carotenoid increased peroxisome proliferator-activated receptor γ levels in THP-1 macrophages. Taken all together, these data bring new information on the anti-atherogenic properties of lycopene, and on its action mechanisms in atherosclerosis prevention [75].

Among the mechanisms proposed as potentially responsible for the beneficial effects of carotenoids we may consider inhibition of Vascular Smooth Muscle Cells (SMCs) proliferation through regulation of the molecular pathways involved in cell proliferation and apoptosis. In fact, several growth factors, among them platelet-derived growth factor (PDGF), and increased SMCs proliferation, play an important role in the development and progression of CVDs. Lycopene inhibited PDGF-BB induced signalling in SMCs of rats via binding to PDGF-BB and inhibiting of the PDGFBB-SMC interaction as well as PDGFBB-induced SMC proliferation [76].

Another key factor in atherogenesis is intravascular thrombosis [77]. Lycopene inhibited both aggregation in human platelets in a dose-dependent manner and the ATP-release reaction stimulated by agonists such as collagen or arachidonic acid. These results may suggest that tomato-based foods might be especially beneficial in the prevention of platelet aggregation and thrombosis [78].

The majority of the discussed *in vitro* studies [65, 68, 76, 78] used very high lycopene concentrations up to 20 mM, which is an undue amount physiologically. When using lower concentrations between 0.5 and 2 mM, an unphysiologically long treatment of up to 24 h [73] was used.

In this connection, our recent results on HUVECs treated with high carotenoid concentrations (2.5 mmol/L to 1 mmol/L), strongly indicate that in these experimental conditions all the carotenoids tested are active in suppressing cell proliferation and decreasing cell viability [31], thus demonstrating that unphysiological doses of carotenoids may bring about their *in vitro* effects not specifically, but through a general cytotoxic action.

This evidence also corroborates the hypothesis that *in vivo* supplementation of carotenoids at pharmacological levels may have adverse effects, possibly through their pro-oxidant activity or, in the case of pro-vitamin A molecules, by overactivating retinoid acid-related signalling [7, 79]. However, at lower doses (below 2.5 mmol/L) we found that both carotenoids reduced the U937–endothelium interaction, confirming their potential beneficial effects in reducing vascular inflammation.

In order to elucidate the mechanism/s potentially underlying the effects of carotenoids, we considered their role in modulating NO bioavailability, according to the evidence that an increased release of this molecule leads to down-regulation of the expression of NF-kB-dependent adhesion molecules in endothelial cells [29, 80].

In our study, we demonstrate that in a model of vascular inflammation, the presence of ‘‘physiological’’ concentrations of β-carotene and lycopene is associated with a significant increase in NO level and its bioavailability (as indicated by the increase in cGMP levels).

As expected, TNF-α treatment led to a fall in NO availability and release due to the reduction of eNOS phosphorylation and to an increase in ROS generation, inducing a situation of endothelial nitro-oxidative stress [81]. The inactivation of NO by ROS, in

particular $O2^-$, is hence recognized to be a crucial factor in reducing NO bioavailability [67]. In this respect, we demonstrated that in cultured HUVECs, either β-carotene or lycopene (2.5 mmol/L, 2 hours) suppressed the increase in ROS generation and the intracellular levels of NT (an index of $ONOO^-$ formation) due to TNF-α treatment. This activity significantly quenched the oxidative stress generated by an inflammatory condition, allowing NO to exert its biological effects, as documented by increased cGMP levels. Note that the increase in NO levels associated with β-carotene and lycopene treatment was not affected by the presence of the eNOS inhibitor L-NAME, indicating that their effect was not due to any specific enzymatic activation of eNOS.

We propose that the candidate mechanism/s potentially responsible for the positive modulation of NO bioavailability by β-carotene and lycopene is associated with their reducing potential. Carotenoids are known to be able to directly interact with several free-radical species *in vitro* [7], and this may account for their radical quenching or scavenging properties. In pro-oxidant conditions, such as in the presence of TNF-α, reducing molecules might contribute to maintaining NO availability by directly interacting with $O2^-$ and therefore minimizing its reaction with intracellular NO and the formation of the potent oxidant-nitrosylating agent, peroxinitrite.

In conclusion, our results, obtained in human endothelial cells exposed to a physiological concentration of carotenoids, proved similar to those occurring in the vessels of subjects consuming a ‘‘normal’’ diet, which provides robust evidence that these molecules may act on an inflammatory vascular state by increasing vascular NO bioavailability thanks to their reducing activity. This looks like another interesting mechanism to further elucidate why carotenoids can prevent and/or delay cardiovascular disease.

Although most of the effects shown in *in vitro* studies cannot be directly transferred to the *in vivo* situation, these findings offer an opportunity to understand the mechanisms underlying the beneficial cardiovascular effects of carotenoids observed *in vivo*.

## *In Vivo* Studies

### Animal Studies

In the last few years, several studies have been conducted on a variety of animal models in order to better understand whether diet supplementation with carotenoids, administered alone or in combination with other molecules of nutritional interest, could exert the hypothesized protective cardiovascular effects in animals.

Bansal et al. [82] fed adult male Wistar rats with lycopene dissolved in olive oil for 31 days. Lycopene reduced levels of lipid peroxides and augmented glutathione levels as well as glutathione peroxidase (GSHPx) activity. The study is thus an example where lycopene reduced oxidative stress in rats, in contrast to other studies where it failed.

In another experiment with female Wistar rats, lycopene was given for 2 week (0.001 and 0.1 g/kg body weight/day). The activity of glutathione reductase, GSH-Px and super oxid dismutase (SOD) was significantly induced by various different doses of lycopene. In contrast, that of catalase (CAT) was not modified [83].

Gitenay et al. [84] explored the potential role of yellow tomato, red tomato or lycopene beadlets in a rat model with mild oxidative stress induced by a diet low in vitamin E. Six week of feeding with 16% freeze-dried yellow tomato, 16% freeze-dried red tomato or 0.05% lycopene beadlets did not affect the cholesterol concentration in plasma. Red tomato intervention decreased triacylglycerol levels compared to controls, yellow tomato and lycopene beadlets. Moreover, thiobarbituric acid-reactive substances levels in the heart were lower after feeding on red tomatoes and yellow tomatoes, as compared to controls and beadlets. Thus, tomatoes had a higher potential than lycopene to affect oxidative stress-related parameters, possibly due to the synergy of all the phytochemicals in tomatoes.

Hsu et al. characterized the lipid-lowering effects and antioxidant mechanisms of tomato paste (t. p., containing approx. 0.1% lycopene) in another animal experiment, this time hamsters. Following 8 weeks of feeding, the authors observed significantly reduced contents of total cholesterol and LDL cholesterol in serum due to feed containing 9% tomato paste. Conversely, High Density Lypoproteins (HDL) cholesterol increased by 19.4% (3% t. p.) or by 28.8% (9% t. p.). In addition, MDA in plasma was reduced by 80.2% (3% t. p.) or by 89.3% (9% t. p.). Regarding the antioxidant enzymes, the activities of CAT, SOD and GSH-Px turned out to have significantly increased after 8 weeks feeding on 9% t. p. [85].

Astaxanthin is a carotenoid with antioxidant, anti-cancer and anti-inflammatory properties. After the intravenous (5, 10 and 20 mg/kg) and oral (100 and 200 mg/kg) administration of astaxanthin, its pharmacokinetic parameters proved dose-dependent and dose-independent, respectively, in rats. The absorption of astaxanthin after oral administration followed the flip-flop model. The hepatic and gastrointestinal first-pass extraction ratios were approximately 0·490 and 0·901, respectively. Astaxanthin was unstable up to a 4 h incubation in the rat gastric juices and 24 h incubation in various buffer solutions having a pH of 1–13 [86].

Recently several studies have suggested a cardiovascular protective role by this compound. Khan SK et al. [87] studied the effect of a proprietary astaxanthin prodrug (CDX-085) on thrombus formation, using a mouse model of arterial thrombosis. The influence of free astaxanthin, the active drug in CDX-085, on human endothelial cells and rat platelets was evaluated to investigate its potential action mechanisms. When compared to control mice, the CDX-085 fed group exhibited significant increases in basal arterial blood flow and significant delays in occlusive thrombus formation following the onset of vascular endothelial injury. Primary human umbilical vein endothelial cells and platelets isolated from Wistar-Kyoto rats treated with free astaxanthin demonstrated significantly increased levels of released NO and significantly decreased peroxynitrite levels. Thus, this study supports the potential of CDX-085 and its metabolite astaxanthin to treat or prevent thrombotic cardiovascular complications. Interestingly, astaxanthin-enriched diet reduces blood pressure and improves cardiovascular parameters in spontaneously hypertensive rats. These effects are accompanied by a decrease in oxidative stress and improvements in NO bioavailability [33]. The ameliorative effect of astaxanthin was again recently demonstrated on endothelial dysfunction in streptozotocin-induced diabetes in rats where it inhibited the ox-LDL-LOX-1-eNOS pathway. This indicates that treatment with astaxanthin might be clinically useful for diabetic complications associated with endothelial dysfunction [88]. Conversely, Jacobsson et al. [89] evaluated the influence of alpha-tocopherol and astaxanthin on LDLoxidation lag time and atherosclerotic lesion formation in Watanabe heritable hyperlipidemic (WHHL) rabbits. They concluded that alpha-tocopherol but *not* astaxanthin prolonged the LDL oxidation lag time.

The two antioxidative substances did not prevent atherogenesis in WHHL rabbits in this setting.

Verghese et al. [90] recently demonstrated that dietary lycopene has a protective effect on cardiovascular disease in New Zealand male rabbits. Animals were fed for 12 weeks on a normal diet, a high cholesterol (5 g/kg) diet and a high cholesterol diet containing various amounts of lycopene. The highest lycopene dose reduced serum cholesterol by 42.8%, increased HDL cholesterol levels and reduced HMG-CoA reductase activity as well as acyl-CoA-cholesterolacyltransferase activity. Of note, the highest dose of lycopene significantly reduced a plaque area in the aorta by 64.3%. Recently, Lee et al. [91] showed the inhibitory effects of lycopene on HMGB1-mediated pro-inflammatory responses in both cellular (HUVECs) and mouse animal models, thereby suggesting its usefulness for vascular inflammatory disease.

The comparison of lycopene and fluvastatin effects on atherosclerosis induced by a high-fat diet in 40 adult male New Zealand white rabbits was lately published by Hu and collegues [92]. The high-fat diet led to increased levels of total cholesterol, total triacylglycerol, LDL cholesterol and IL-1. Lycopene (8 wk) was better than fluvastatin in reducing the changes in these parameters. Lycopene and fluvastatin also markedly reduced the formation of atherosclerotic plaques in the aorta compared to the situation in rabbits on a high-fat diet alone [92].

In contrast, Frederiksen et al. [93] did not show any effects from an intervention (16 wk) with extract of lycopene rich tomatoes when investigating 65 male Watanabe heritable hyperlipidemic rabbits. They fed on a control diet, a control diet supplemented with a mixture of plant oils or a control diet supplemented with tomato extract (0.25 g tomato extract (containing 6% lycopene)/100 g:15mg lycopene/ 100 g diet). The tomato extract had no effect on cholesterol and triacylglycerol levels in plasma, on cholesterol in lipoprotein fractions and on aortic atherosclerosis (cholesterol in tissue, microscopy). Oxidation of plasma lipids was also unaffected by the intake of tomato extract. These results were recently confirmed by Lorenz M et al. [94]. In fact, although they found that lycopene supplementation for 4 weeks increased its plasma levels and strongly reduced total and LDL cholesterol serum levels as well as significantly lowering amounts of cholesteryl ester in the aortae in lycopene-treated New Zealand white rabbits, no significant differences in initial lesions to the aorta were detected.

One notable recent study demonstrates that a dietary mix of fish oil, resveratrol, lycopene, catechin, d-α-tocopherol, and vitamin C, which was shown to be well tolerated in humans, improves lipid and inflammatory risk factors for CVD in humanized models of disease [95]. These findings support the concept of combination strategies with several bioactive nutrients and a systems-based, multi-target approach for complex multifactorial diseases, such as type 2 diabetes. In this regard, Zhu J et al. [96] demonstrated that chronic lycopene treatment could attenuate endothelial dysfunction by reducing oxidative stress in streptozotocin-induced diabetic rats, indicating that chronic lycopene treatment might be useful in preventing the diabetic vascular complications associated with endothelial dysfunction.

In conclusion, taking into account that most such animal experiments [82-85, 90, 92, 93] were carried out to evaluate the possible beneficial effects of dietary lycopene in humans, we can assume, first, that the intervention periods, lasting between 2 and 16 weeks, are also relevant to the human situation. Secondly, the lycopene dosage may be a critical parameter.

The authors used lycopene dosages between 0.001 mg/kg b.w. [83] and 127.8 mg/kg diet [90].

As the animals partly had free access to the feed (e.g. [90]) and otherwise got a restricted amount (e.g. 100 g: [93]), the studies are not comparable. Experiments with a lycopene dose of approx. 10 mg/kg b.w. cannot be transferred to the human situation. In addition, it must be considered that experiments with rats [82-84], hamsters [92] and rabbits [90, 92, 93] cannot be directly converted into the conditions found in the human organism.

## Human Intervention Studies

In recent times, Yang et al. [97] reviewed the major publications relating to the potential effects on cardiovascular risk factors and outcomes of some common dietary constituents: carotenoids, flavonoid-rich cocoa, tea, red wine and grapes, coffee, omega-3 fatty acids, and garlic. Increased intake of some of these has been associated with reduced all-cause mortality or reduced incidence of myocardial infarction, stroke, and hypertension. However, although the evidence from observational studies, *in vitro* studies and animal studies showed linkages between carotenoids and prevention of cardiovascular disease and is supportive of beneficial effects for most of these foodstuffs taken as part of the diet, the potential benefits of using supplements derived from these natural products remain largely inconclusive.

The fact is that although many epidemiological studies have reported an association between β-carotene and the risk of CVD, several large randomized trials failed to reveal any reduction in CVD with β-carotene consumption. For instance, the MRC/BHF Heart Protection Study showed no benefit from β-carotene 20 mg daily, in combination with vitamin E 600 mg and vitamin C 250 mg, on morbidity or mortality in high-risk individuals [98]. In the α-tocopherol and β-carotene (ATBC) study conducted on 1,862 male smokers who had had a previous myocardial infarction, there were no significant differences in the number of major coronary events between any supplementation group and the placebo group. Moreover, the risk of fatal CHD was increased in the β-carotene and combined α-tocopherol and β-carotene groups as compared to the placebo group [99]. Likewise, the Women's Antioxidant Cardiovascular Study (WACS) found no CVD risk reduction in women at high risk, whether using β-carotene 50 mg every other day, or vitamin C 500 mg daily or vitamin E 600 IU every other day [100]. The prospective evaluation of the relation between vegetable intake and CHD risk in the Physicians' Health Study concluded that the consumption of vegetables rich in carotenoids was associated with a reduced risk of CHD [101], but after 12 years of follow-up there was no impact from supplementation of alternate day β-carotene 50 mg on CVD, cancer, or overall mortality among primarily non-smokers [102].

Moreover, in the Physicians' Health Study, no association between increasing concentrations of plasma lycopene and the risk of CVD was found [103]. A recent review of the controlled clinical studies with lycopene in well-defined subject populations found no definite evidence for CVD prevention [104].

To compare the effect of lutein- and zeaxanthin-rich foods and supplements on macular pigment levels (MPL) and serological markers of endothelial activation, inflammation and oxidation in healthy volunteers, Graydon R et al. [105] conducted two 8-week intervention studies concluding that this 8-week supplementation with lutein and zeaxanthin, whether as foods or as supplements, had no significant effect on MPL or serological markers of

endothelial activation, inflammation and oxidation in healthy volunteers, but may improve MPL in the highest serum responders and in those with initially low MPL.

The effects of lycopene (from cooked tomatoes) on serum antioxidant enzymes, lipid peroxidation rate and lipid profile were evaluated in a case-control study involving 20 coronary heart disease patients. They were asked to eat 200 g cooked tomatoes every day for 60 days. Supplementation with tomatoes significantly reduced MDA levels, indicating a lower rate of lipid peroxidation. and conversely increased levels of antioxidant enzymes (SOD, glutathione reductase, GSH-Px), while lipid status parameters were not affected [106]. The same authors studied the effect of lycopene from cooked tomatoes on plasma antioxidant enzymes, lipid peroxidation rate and lipid profile in grade-I hypertension, and concluded that tomato lycopene may have considerable natural therapeutic potential as an antioxidant but may not be used as a hypolipidemic agent in hypertension [107]; they demonstrated that a relatively high daily consumption of tomato-based products (equivalent to 32-50 mg lycopene/d) or lycopene supplements (10 mg/d) is ineffective in reducing conventional CVD risk markers in moderately overweight, healthy, middle-aged individuals.

The effects of a tomato-based drink on markers of inflammation, immunomodulation, and oxidative stress were studied in a randomised, placebo-controlled, double-blind, crossover study. 26 healthy men and women were supplemented with placebo and tomato-based drink (ingesting 5.7 mg lycopene per day) for 26 days per period. The tomato-based drink significantly reduced TNF-α production in challenged whole blood. By contrast, DNA damage and urinary 8-iso-PGF2α concentration were not affected by tomato drink consumption [37]. Effects of lycopene supplementation on oxidative stress and markers of endothelial function in healthy men were recently published in another randomised, placebo-controlled, double-blind study [108]. Supplementation of 126 healthy men (22–57 years), with 6 or 15 mg lycopene daily for 8 weeks led to significantly increased lycopene contents in serum. As was shown by decreased DNA damage as well as by increased SOD activity, oxidative stress was reduced by lycopene supplementation. Endothelial function, evaluated by the reactive hyperemia peripheral arterial tonometry index, was significantly improved by lycopene (15 mg per day). This dosage also significantly decreased the hs-CRP content in serum, a marker of inflammatory status. Plasma levels of adhesion proteins sICAM- 1 and sVCAM-1 were significantly decreased by lycopene. Thus, supplementation with 15 mg lycopene per day over 8 weeks was able to reduce oxidative stress as well as to improve endothelial function. This study specially focused on middle-aged Korean men; hence the results cannot be generalised to women. However, the results demonstrated the antioxidative and anti-inflammatory effects of lycopene [108].

By way of contrast, one recent intervention with 70 g tomato purèe per day (46 mg lycopene per day) in a group of 31 non-smoking healthy postmenopausal women did not affect endothelial function. Though the concentration of lycopene in plasma significantly increased, flow-mediated dilation did not change during the intervention period [109].

A meta-analysis, employing human intervention trials between 1955 and September 2010, investigated the effect of lycopene on blood lipids and on blood pressure [110]. Ried and Fakler's meta-analyses indicated that 25 mg daily of lycopene effectively reduced total cholesterol and LDL cholesterol in serum. Regarding the potential role of lycopene in the regulation of blood pressure, the clinical trails published to date are too low in number (54) to offer any firm evidence of this. Although they do suggest lycopene has a lowering effect on

systolic blood pressure, in particular in hypertensive subjects, further studies are necessary to prove these results.

Astaxanthin, a xanthophyll carotenoid, is a nutrient with unique cell membrane actions and diverse clinical benefits, with excellent safety and tolerability. Significant antioxidant powers have been ascribed to astaxanthin, based primarily on experimental findings. The real breakthrough with this nutrient, however, is that it produces clinically significant antioxidant benefits in human subjects, including groups especially vulnerable to oxidative stress, such as smokers, the obese, and the overweight.

In a Korean prospective, randomized, double-blind study, astaxanthin "normalized" oxidative stress in individuals with weight challenges [111]. In this three-week study, twenty-three adults with Body Mass Index (BMI)> 25.0 kg/m2 enrolled in this study and were randomly assigned to two dose groups: astaxanthin 5 mg and 20 mg once daily for 3 weeks, and compared to a control group (n=10) with normal body weight (BMI <25.0 kg/m2) who received no intervention. Malondialdehyde, isoprostane, superoxide dismutase and total antioxidant capacity, as oxidative stress biomarkers, were measured at baseline and 1, 2 and 3 weeks after astaxanthin administration. Compared with baseline, the malondialdehyde (by 34.6% and 35.2%) and isoprostane (by 64.9% and 64.7%) levels were significantly lowered, whereas superoxide dismutase (by 193% and 194%) and total antioxidant capacity (by 121% and 125%) levels were significantly increased in two dose groups after the 3 week intervention. This study revealed that supplements of astaxanthin for 3 weeks improved oxidative stress biomarkers by suppressing lipid peroxidation and stimulating the activity of the antioxidant defense system.

Another double-blind, randomized controlled trial was conducted by the same group [112]. Thirty-nine heavy smokers (≥20 cigarettes/day) and 39 non-smokers were enrolled in this study. Smokers were randomly allocated to receive astaxanthin at 5-, 20-, or 40 mg/day for three weeks. Oxidative stress biomarkers such as malondialdehyde, isoprostane, superoxide dismutase, and total antioxidant capacity, and astaxanthin levels in plasma were measured at baseline and after 1, 2, and 3 weeks of treatment. Compared with baseline, the plasma malondialdehyde and isoprostane levels decreased, whereas superoxide dismutase levels and total antioxidant capacity increased in all astaxanthin intervention groups over the 3-week period. In particular, isoprostane levels showed a significant dose-dependent decrease after astaxanthin intake. The results suggest that astaxanthin supplementation might prevent oxidative damage in smokers by suppressing lipid peroxidation and stimulating the activity of the antioxidant system in smokers.

In the Park double-blind, randomized controlled trial [113], astaxanthin also significantly lowered C-reactive protein, a biomarker of systemic inflammation [114].

Astaxanthin improved certain blood lipids in subjects with moderately elevated serum triglycerides. Healthy non-obese subjects (BMI <28 kg/m2), aged 20-65 years (n=61) with fasting triglycerides in the range 120-200 mg/dL, were recruited into a double-blind randomized controlled trial [115]. They were randomly allocated to receive astaxanthin at 6, 12, or18 mg/day, or a placebo for 12 weeks. Astaxanthin, as compared to placebo, significantly elevated HDL-cholesterol at the doses of 6 mg/day ($p<0.05$) and 12 mg/day ($p<0.01$). It also significantly lowered triglycerides at doses of 12 mg/day and 18 mg/day ($p<0.05$ for both) as compared to placebo. There was no effect on LDL-cholesterol at any dose.

Astaxanthin also significantly increased blood adiponectin levels ($p<0.01$ at 12 mg/day; $p<0.05$ at 18 mg/day). Adiponectin is a hormone produced by adipose tissue, cardiac and skeletal muscle, and vessel endothelia. Serum levels of adiponectin tend to be reduced in obese and/or diabetic subjects, smokers, patients with coronary heart disease, and individuals with metabolic syndrome [116]. Although the results of this study suggest a normalization of adiponectin levels, 12 weeks of supplementation had no effect on BMI. Further investigation is required under better controlled conditions in order to clarify astaxanthin's utility for this condition.

As reviewed by Fasset and Coombes [117], the safety, bioavailability and effects of astaxanthin on oxidative stress and inflammation that have relevance to the pathophysiology of atherosclerotic cardiovascular disease have been assessed in a small number of clinical studies. No adverse events have been reported and there is evidence of a reduction in biomarkers of oxidative stress and inflammation with astaxanthin administration. Experimental studies in several species using an ischaemia-reperfusion myocardial model demonstrated that astaxanthin protects the myocardium when administered both orally or intravenously prior to induction of the ischaemic event. At this stage we do not know whether astaxanthin is of benefit when administered after a cardiovascular event and no clinical cardiovascular studies in humans have been completed and/or reported. Cardiovascular clinical trials are recommended and warranted, judging by the physicochemical and antioxidant properties, the safety profile and preliminary experimental cardiovascular studies on astaxanthin.

As widely reviewed by Bohm [59], most intervention studies published, especially those conducted using lycopene [37, 106, 108, 109], were perfomed on healthy subjects. Thus, the authors of these studies investigated the possible primary preventive effect of lycopene contained in tomatoes or their products. Some studies investigated only men [108] or women [109] while others used men and women as volunteers [37, 106]. This renders a comparison of the investigations complex. In addition, the duration of intervention trials also varied between 7 days [109] and 8 weeks [108] and the authors used lycopene dosages from 5.7 [37] up to 46.2 mg per day [109]. Another factor affecting the results could be the matrix of the intervention products, such as, tomato-based drink, tomato oleoresin capsules, raw tomatoes and tomato purée. Thus, no direct comparison among the studies is conceivable.

## Conclusion

Increasing evidence indicates that an appropriate redox balance may be implicated in preserving health and longevity. Altering this equilibrium in favour of oxidants may result in pathological responses causing functional disorders and disease. Promising epidemiological studies on nutrition, associating high levels of carotenoids with low levels of oxidative stress (oxLDL, conjugated dienes, *etc.*) establishes that higher fruit and vegetable intakes tend to be associated with lower rates of heart and vascular diseases, including coronary heart disease and stroke. Notably, short-term dietary intervention trials suggest that those who consume higher amounts of fruits and vegetables tend to have improvements in coronary risk factors and reduced cardiovascular mortality.

Carotenoids are a large family of pigmented compounds that are synthesized by plants and micro-organisms but not animals. In human diet fruit and vegetables constitute the main sources of carotenoids, fat-soluble pigments that can function as antioxidants. Although more than 600 carotenoids have been identified, most research in nutrition has focused on the five most common carotenoids: α-carotene, β-carotene, lycopene, lutein/zeaxanthin, and β-cryptoxanthin. In addition, astaxanthin is a xanthophyll carotenoid present in micro-algae, fungi, complex plants, seafood, flamingos and quail. It is an antioxidant with anti-inflammatory properties and as such has potential as a therapeutic agent in atherosclerotic cardiovascular disease.

As discussed earlier, carotenoids may prevent cardiovascular disease in a number of ways. For these reasons, carotenoids from plants may represent one possible mechanism by which fruit and vegetables reduce the risk of heart and vascular disease.

Previously, carotenoids were known to exert relevant beneficial properties on human health. Their biological role in the prevention and possibly the treatment of cardiac and vascular diseases is now being studied and partially understood.

Epidemiological and human intervention studies have verified the significance of these natural molecules in the prevention of human diseases. *In vitro* and animal investigations have tested the hypotheses generated from the epidemiological reports. Although some human clinical trials are beginning to be undertaken, there is a great need for well-designed human intervention studies designed to define subject selection, end point measurements and the levels of carotenoids being tested. It is only through such studies that our knowledge of the crucial role played by carotenoids will improve and enable us to develop complementary approaches to the prevention, cure and management of cardiovascular diseases.

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