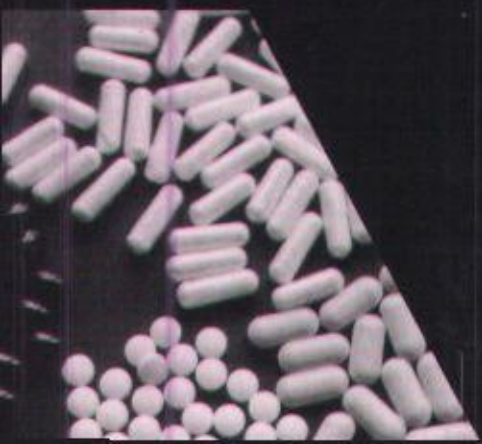
# Antioxidants and air pollution in relation to indicators of asthma and COPD



Linda Grievink

# STELLINGEN

1. De antioxidant β-caroteen heeft wel vitamine A activiteit, maar vitamine A heeft nauwelijks antioxidant activiteit.

2. Vitamine E concentraties in de voeding en in bloed zijn niet in gunstige richting geassocieerd met CARA-indicatoren. (*dit proefschrift)*

3. Een goede β-caroteen status of suppletie met antioxidanten biedt gedeeltelijke bescherming tegen acute effecten van luchtverontreiniging. (*dit proefschrift*)

4. Kortademigheidsklachten zijn geassocieerd met risicofactoren voor hart- en vaatziekten. (*dit proefschrift*)

5. Om de resultaten tussen onderzoeken te vergelijken is een kwantitatieve schatting van de sterkte van de associaties belangrijker dan de significantie niveau's van deze associaties.

6. Kwaliteitsbewaking is essentieel bij gegevensverzameling in grootschalig epidemiologisch onderzoek.

7. Voor een goede interpretatie van onderzoeksresultaten is het noodzakelijk dat onderzoekers zelf betrokken zijn bij de gegevensverzameling.

8. Alhoewel in de epidemiologie van zowel voeding als luchtverontreiniging kleine gezondheidseffecten worden gevonden kunnen deze grote gevolgen voor de volksgezondheid hebben.

9. De bewering in de reclame dat antioxidanten in huidverzorgingsprodukten de veroudering van de huid kunnen tegengaan, berust niet op gedegen wetenschappelijk onderzoek en is daarom misleidend.

10. Vergaderingen verlopen efficiënter als ze op vrijdagmiddag worden gehouden.

11. 'Mastermind' is binnen 5 beurten op te lossen mits er verschillende kleuren worden gebruikt.

12. Geen tijd is geen prioriteit.

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# Antioxidants and air pollution in relation to indicators of asthma and COPD

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# Antioxidants and air pollution in relation to indicators of asthma and COPD

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# ABSTRACT

Two main research questions were specified in this thesis. First, whether acute respiratory effects of air pollution can be modulated by antioxidants. Second, whether dietary or plasma antioxidants were associated with indicators of asthma and COPD.

Two intervention studies investigated a possible modulation of the acute respiratory effects of ozone by antioxidant supplementation. In addition a panel study examined a possible modulation of the acute respiratory effects of winter air pollution by antioxidants in diet and serum. The first intervention study in 1994 was a pilot study among 26 cyclists who performed lung function measurements (192 observations) before and after exercise. Half of the group was randomly assigned to the supplementation group and were given a daily antioxidant supplementation of vitamins C, E and β-carotene. The control group did not receive a placebo. We repeated the study in the summer of 1996 with a similar design but this time the study was placebo-controlled. In this study, 38 subjects (380 lung function measurements) participated until the end of the study and the antioxidant supplementation consisted of a cocktail of vitamins C and E. Both intervention studies suggest that there was an effect of ozone on $FEV_1$ and FVC in the control group. There was no change in lung function when ozone levels were high in the supplementation group. The difference in ozone effect between the groups for both studies was statistically significant for $FEV_1$ and FVC. In the analysis of the panel study, we included only subjects with chronic respiratory symptoms because these subjects showed clear acute respiratory effects of air pollution. The results suggest that subjects with low levels of plasma β-carotene showed an effect of air pollution on large PEF decrements, in particular, for PM10 and black smoke, whereas subjects with high levels of plasma β-carotene did not show an effect of air pollution. No difference in acute respiratory effects of air pollution was observed for a high versus a low dietary intake of vitamin C, E and β-carotene or for plasma α-tocopherol.

The second research question was investigated within the MORGEN study. This study is a cross-sectional investigation on the prevalence of risk factors for chronic diseases using self-administered questionnaires and a physical examination in a randomly selected sample of the Dutch population. First, we examined the relations between dietary antioxidants (vitamins C, E and β-carotene) and the prevalence of a number of respiratory symptoms and lung function in a population based sample of 6,555 adults. Our results suggested that a high dietary vitamin C and β-carotene intake was associated with a higher $FEV_1$ and FVC. Dietary vitamin E was not associated with lung function. None of the dietary antioxidants were consistently associated with the prevalence of a number of respiratory symptoms. Second, we studied the relation between plasma levels of β-carotene or α-tocopherol and respiratory symptoms in a case-control sample of never and long-term former smokers. Our results suggested that cases (subjects with one or more chronic respiratory symptoms; n=491) tended to have lower plasma β-carotene levels than controls (n=496). Plasma α-tocopherol was not associated with asthma and chronic bronchitis symptoms but was positively associated with dyspnea. This adverse association of plasma α-tocopherol could not be explained by adjustment for cardiovascular risk factors and remains puzzling. Third, we evaluated the relation between plasma antioxidants (β-carotene and α-tocopherol) and lung function in a random sample (n=367) of the MORGEN study. We found that subjects with a high plasma β-carotene concentration tended to have a higher FVC and $FEV_1$ than subjects with a low plasma β-carotene concentration but this was not statistically significant for $FEV_1$. Plasma α-tocopherol was not associated with lung function. In conclusion, an elevated antioxidant status suggests to protect partly against respiratory effects of air pollution. More research is needed to investigate if antioxidants (vitamin C, β-carotene) are really the beneficial agents in relation to (indicators of) asthma and COPD.

# CONTENTS

# CHAPTER 1

## General Introduction

## Background

There is a growing recognition that air pollution even at low levels could have adverse health effects [1]. Several epidemiological studies suggested that air pollutants, such as ozone and particulate matter are related to acute respiratory effects, such as a decrease in pulmonary function and an increase in respiratory symptoms [2-6]. There is also an increasing recognition that the incidence and the rate of mortality of asthma have been increasing in the Netherlands and in other Western countries [7,8]. Additionally, Chronic Obstructive Pulmonary Disease (COPD) is currently one of the important chronic diseases [9]. For both the acute respiratory effects of air pollution and the occurrence of asthma and COPD, oxidant processes and free radical reactions are involved in the mechanisms. Therefore, antioxidants have been suggested to be protective factors in relation to acute respiratory effects of air pollution [10-12] and in relation to chronic conditions, such as asthma and COPD [13-23].

A possible modulation of the acute respiratory effects of short-term exposure of air pollution by antioxidants has been suggested in some small experimental studies [10-12]. Antioxidants have also been investigated in relation to indicators of asthma and COPD, such as lung function [13-15], respiratory disease or symptoms [16-19] and bronchial reactivity [20-23].

Two main research questions were (1) can antioxidants modulate the acute respiratory effects of air pollution under ambient conditions and (2) is there a relation between antioxidants (in diet and blood) and respiratory symptoms and lung function.

## Antioxidants

What are antioxidants? Antioxidants are defined by their mechanistic action. Antioxidant mechanisms are any cell process that (1) prevents the formation of free radicals, (2) converts oxidants to less toxic species, (3) separates reactive species away from vital cellular structures, or (4) repairs oxidant injury [24]. As a first line of defence in the lungs, there are the antioxidant enzymes, such as superoxide dismutases which convert superoxide anion radicals to hydrogen peroxide, catalase which reduces hydrogen peroxide to water [25], and enzymes of the glutathione redox cycle, such as glutathione peroxidase which is selenium dependent. In the second line of defence operates uric acid and the antioxidant (pro)-vitamins, such as vitamins E, C and β-carotene that are derived from diet [24]. Uric acid scavenges hydroxyl, superoxide, and peroxyl radicals [25] and prevents the oxidation of vitamin C.

Vitamin C or ascorbic acid is water-soluble and is mainly derived from fruits, vegetables and potatoes in the Dutch diet. It is present in both extracellular and

intracellular fluids in the lungs. Vitamin C can scavenge oxygen radicals and neutralises the extracellular oxidants released from stimulated neutrophils [24].

Vitamin E comes mostly from vegetable oils and margarines in the Netherlands and consists of four lipid-soluble tocopherols (α, β, δ, γ-tocopherol). α-Tocopherol is the most active antioxidant of the four tocopherols [26] and is present in the lipid membranes and in the extracellular fluids. It can convert oxygen radicals and lipid peroxyl radicals to less reactive forms and is in this way able to break the lipid peroxidation chain reaction. The formed α-tocopherol radical can be reduced back to a α-tocopherol molecule by reduction of ascorbic acid [27].

β-Carotene is a pro-vitamin A and is mostly present in vegetables. It is a lipid soluble antioxidant available in membranes of tissues [24] and it is able to scavenge superoxide anion and peroxyl radicals [25]. A synergistic interaction between α-tocopherol and β-carotene has been suggested *in vitro* with an increased inhibition of lipid peroxidation in rat liver microsomes [28].

## Evidence: antioxidants in relation to the acute effects of air pollution

Table 1 summarises the design and results of studies on modulation of acute respiratory effects of air pollution by antioxidant supplementation. Some of these studies used controlled exposure to single pollutants. Other studies were conducted under ambient conditions of exposure to pollutant mixtures.

The first experimental studies have investigated a possible protective role of antioxidants on acute effects of ozone and $NO_2$. The first two experiments did not find a protective role of supplemental vitamin E alone for 10 to 12 weeks on lung function or respiratory symptoms after exposure to a single exposure of 1,000 $\mu g/m^3$ ozone for 2 hours while the subjects were exercising [29]. However, a combination of supplemental vitamin C and vitamin E was shown to protect partly against acute effects of ozone (600 $\mu g/m^3$) for 2 hours with exercise on lung function [10]. The acute effects of $NO_2$ on lipid peroxidation were measured in one study [30] and on hyperresponsiveness to metacholine in another study [12]. After supplementation with a cocktail of vitamin C and E, subjects had lower levels of lipid peroxidation products in lung lavage and higher levels of $\alpha_1$-antitrypsin in the alveolar lining fluids after exposure to 7,520 $\mu g/m^3$ $NO_2$ compared to subjects in the placebo group [30]. Vitamin C supplementation alone showed an improvement of airway responsiveness after exposure to $NO_2$ (3,760 $\mu g/m^3$) compared to placebo [12]. No exercise was performed during exposure in both studies.

**Table 1:** *Evidence of acute effects of air pollution being modulated by antioxidant supplements*

| Study | Design | Subjects | Exposure | Suppl. | Outcome | Asso-ciation |
|---|---|---|---|---|---|---|
| Hackney et al [29] | double-blind* | 34 healthy adults | 2-h 1000 μg/m$^3$ $O_3$ + exercise | 0.8 g/day vit E for 10 wks | symptoms & lung function | - |
| | double-blind | 22 healthy adults | 2-h 1000 μg/m$^3$ $O_3$ + exercise | 1.6 g/day vit E for 12 wks | symptoms & lung function | - |
| Chatham et al [10] | double-blind cross-over | 14 healthy adults | 2-h 600 μg/m$^3$ $O_3$ + exercise | 1 g vit C once 0.8 g/day vit E for 2 wks (?) | lung function | + |
| Mohsenin et al [12] | double-blind cross-over | 11 healthy adults | 1-h 3760 μg/m$^3$ $NO_2$ | 0.5 g vit C 4 times /day for 3 days | airway reactivity to metacholine | + |
| Mohsenin et al [30] | double-blind | 19 healthy adults | 3-h 7520 μg/m$^3$ $NO_2$ | 1.5 g vit C/day 1.2 g vit E/day for 4 weeks | lipid peroxidation & $\alpha_1$-antitrypsin in alveolar fluids | + |
| Bucca et al [11] | double-blind cross-over | 20 healthy policemen | 2-h traffic directing in city during winter | 2 g vit C once | $MEF_{50}$ & airway reactivity to histamine | + |
| | double-blind cross-over | 20 healthy policemen | 4 days traffic directing in city during winter | 2 g vit C/day for 4 days | PEF | + |
| Romieu et al [31] | double-blind cross-over | 49 street workers | mean ambient level $O_3$ 163 μg/m$^3$ | daily 650 mg vit C, 75 mg vit E, 15 mg β-carotene | repeated lung function measurements | + |
| Trenga et al [32] | double-blind cross-over | 17 asthma patients | 45 min 240 μg/m$^3$ $O_3$ + exercise | 500 mg vitC, 400 mg vit E duration? | PEF | + |
| Samet et al [33] | double-blind placebo | 30 healthy adults | 2-h 800 μg/m$^3$ $O_3$ + exercise | 2-w daily 250 mg vit C, 100 mg vit E, vegetable cocktail | $FEV_1$, FVC | + |
| | | | | | Inflammatory markers (BAL) | - |

* The term double blind denote placebo-controlled

Suppl.: Supplementation; Association: - = no association; + = beneficial association

There has only been one small study investigating a possible modulation of the acute effects of ambient winter air pollution on lung function by vitamin C in two experiments [11]. During winter concentrations of ozone are usually low, whereas concentrations of particulate matter tend to be higher. The exact levels of exposure to air pollutants were, however, unclear in this study. In the first experiment, vitamin C supplementation was suggested to prevent a decrease in lung function ($MEF_{50}$)

and airway responsiveness to histamine. In the second experiment, vitamin C was supplemented and peakflow (PEF) measurements before and at the end of the working day were performed in policemen during four consecutive days of traffic directing. In this experiment, the PEF decreased during the working day in the control group but not in the vitamin group.

Most of these earlier studies were under experimental conditions investigating one pollutant during only one or two exposures and with a small number of subjects. The levels of ozone and $NO_2$ were very high in these studies. All of these studies were supplementing subjects for short periods with high doses of vitamin C or E. These doses were at least eight to 16 times the US Recommended Dietary Allowances (RDA) of vitamin C (60 mg/day) and 100 times the RDA of vitamin E (8 mg/day for women and 10 mg/day for men) [34].

Recent studies have addressed conditions in which the levels of ozone exposure and the doses of the supplements were mostly lower. One experimental study was performed in asthmatics [32]. After a 45 min of exposure to ozone (240 $\mu g/m^3$) with exercise, subjects were challenged with two concentrations of $SO_2$ (0.10 & 0.25 ppm) for 10 minutes. Preliminary results suggest that a cocktail of vitamin C and E protected against PEF decrements from $SO_2$ challenges after ozone exposure in only the group of severe asthmatics (n=6). The duration of supplementation in this study was unclear. The other recent experimental study exposed healthy subjects to relatively high levels of ozone (800 $\mu g/m^3$) for 2 hours while the subjects were exercising [33]. Subjects were placed on a diet low in vitamin C for 3 weeks and after that they were supplemented for 2 weeks with a relatively low level of vitamin C (250 mg). Preliminary results of the first 18 subjects suggest that a supplemental cocktail of vitamin C, E and vegetables protected against pulmonary function decrements but not against an increase in inflammatory markers. It is unclear what the time was between ozone exposure and broncho-alveolar lavage measurements for determination of the inflammatory markers. Next to the studies described in this thesis, there has been one study investigating modulation of the acute effects of ambient ozone on lung function [31]. This placebo controlled cross-over study was among street workers in Mexico-city and the measurement periods were from half March to end of May and from half June to half August 1996. The results suggest that supplementation of vitamin C, E and β-carotene protected against decrements of $FEV_1$, FVC and PEF, in particular, in the first period. The control group in the second period did not show an ozone effect probably because the subjects had still elevated plasma levels of the antioxidants due to the supplementation in the first period.

## Epidemiological evidence: antioxidants in relation to asthma and COPD

Most studies were observational (prospective or cross-sectional) in design. There has been one intervention study (the alpha-tocopherol beta-carotene cancer prevention study) investigating supplementation of α-tocopherol (50 mg/day) and β-carotene (20 mg/day) for six years among smokers (n=29,133) on the incidence of COPD symptoms; the results showed no effect of this supplementation [19]. Very recently an abstract was published with data from the CARET trial investigating the effect of 11 year supplementation of β-carotene (30 mg/day) and retinyl palmitate (25,000 IU/day) on lung function decline in 3,056 subjects [35]. In this trial the annual decline in lung function did not differ between the supplementation group and the placebo group.

Early publications suggested a beneficial effect of fruit consumption on lung function in a cross-sectional study (n=2,859) [36] and the 25-years incidence of chronic non-specific lung disease in middle-aged men (n=793) [16]. Recently fruit, salad and green vegetable consumption was associated with a higher lung function, but not with the prevalence of wheeze in children (n=2,650) [37]. Vegetable consumption (excluding legumes and potatoes) of more than seven servings per week was associated with a lower prevalence of self-reported chronic bronchitis and bronchial asthma compared to less than seven servings per week in 46,693 subjects aged 15 years or over in Italy [38]. The suggested specific antioxidants in fruits and vegetables that might be responsible for the observed beneficial effect on asthma and COPD are vitamin C, carotenoids and flavonoids [16,36].

The evidence for relations between vitamin C (dietary and blood levels) and (indicators of) asthma and COPD is summarised in Table 2. Dietary intake of vitamin C was not associated with 25-year incidence of CNSLD [16] and 10-year incidence of asthma [17] while serum vitamin C was associated with a lower prevalence of wheeze and chronic bronchitis [18]. Serum vitamin C was, however, not associated with respiratory symptoms in a small study among elderly (n=96) in which the subjects were measured 6 times over one year [39]. In this study, data on respiratory symptoms (not specified) were collected for a relatively short period, that is, the past fortnight. One study investigated bronchial reactivity in relation to dietary antioxidants [22]. This study showed that subjects with an airway hyperreactivity (20% fall in $FEV_1$) after a metacholine challenge had lower dietary intake of vitamin C compared to healthy subjects. In several large (N >1,500) cross-sectional studies, dietary and plasma vitamin C were positively associated with lung function [13,14,40]. Two smaller studies, one in elderly [15] and one in children [37] did, however, not show an association between vitamin C and lung function. In a small study among elderly (6 times measured in one year) a positive association between serum vitamin C and $FEV_1$ was found [39].

**Table 2:** *Evidence of a relation between vitamin C and (indicators of) asthma and COPD*

| Study | Design | No. of subjects | Gender & age range (yrs) | Vitamin C measured in | Outcome | Asso-ciation |
|---|---|---|---|---|---|---|
| Miedema et al [16] | Prosp. | 793 | ♂ 40-60 | Diet | 25-years CNSLD | - |
| Troisi et al [17] | Prosp. | 77,866 | ♀ 34-68 | Diet | 10-years asthma | - |
| Schwartz et al [18] | Cross. | 9,074 | ♂♀ 30-75 | Serum | wheeze | + |
| | | | | | bronchitis | + |
| Khaw et al [39] | Cross. | 96 | ♂♀ 65-74 | Serum | resp. symptoms | - |
| | | | | | $FEV_1$ | + |
| Soutar et al [22] | Cross. | 87 | ♂♀ mean: 37 | Diet | $PD_{20}$ metacholine | + |
| Schwartz et al [40] | Cross. | 2,526 | ♂♀ 30-74 | Diet | $FEV_1$ | + |
| Britton et al [13] | Cross. | 2,633 | ♂♀ 18-70 | Diet | $FEV_1$, FVC | + |
| Ness et al [14] | Cross. | 1,860 | ♂♀ 45-75 | Serum | $FEV_1$, FVC | + |
| Dow et al [15] | Cross. | 178 | ♂♀ 70-96 | Diet | $FEV_1$, FVC | - |
| Cook et al [37] | Cross. | 278 | ♂♀ 9-11 | Serum | $FEV_1$ | - |

Cross. = Cross-sectional; Prosp. = Prospective; ♀ women, ♂ men
Association: - = no association; + = beneficial association

The evidence for relations between vitamin E (diet and blood levels) and (indicators of) asthma and COPD is summarised in Table 3. A high dietary intake of vitamin E was associated with a decreased 6-years incidence of adult-onset asthma [17], a lower prevalence of COPD symptoms in male smokers [19] and a higher lung function in elderly subjects [15]. However, dietary vitamin E was not associated with lung function in a large cross-sectional study after adjustment for vitamin C [13] and with hyperreactivity [22]. Additionally, blood levels of vitamin E were also not associated with the development of airway obstruction during five subsequent years [41].

**Table 3:** *Evidence of a relation between vitamin E and (indicators of) asthma and COPD*

| Study | Design | No. of subjects | Gender & age range (yrs) | Vitamin E measured in | Outcome | Asso-ciation |
|---|---|---|---|---|---|---|
| Troisi et al [17] | Prosp. | 77,866 | ♀ 34-68 | Diet | 6-years asthma | + |
| | | | | | 10-years asthma | - |
| Rautalahti et al [19] | Cross. | 29,133 | ♂ 50-69 | Diet & Serum | COPD symptoms | + |
| Dow et al [15] | Cross. | 178 | ♂♀ 70-96 | Diet | $FEV_1$, FVC | + |
| Britton et al [13] | Cross. | 2,633 | ♂♀ 18-70 | Diet | $FEV_1$, FVC | - |
| Soutar et al [22] | Cross. | 87 | ♂♀ mean 37 | Diet | $PD_{20}$ metacholine | |
| Morabia et al [41] | Prosp. | 83 | ♂ 50-79 | Serum | $FEV_1 \leq 75\%$ FVC | - |

Cross. = Cross-sectional; Prosp. = Prospective; ♀ women, ♂ men
Association: - = no association; + = beneficial association

The evidence for a relation between β-carotene (diet and blood levels) and (indicators of) asthma and COPD is summarised in Table 4. Dietary β-carotene was not associated with 25-year incidence of CNSLD in men [16]. However, dietary β-carotene was borderline significantly associated with a lower 10-year incidence of adult-onset asthma in women [17] and significantly with a lower prevalence of hyperreactivity [22]. Additionally, both serum and dietary β-carotene were associated with a lower prevalence of COPD symptoms in male smokers [19].

The intake of total carotene was not associated with $FEV_1$ and FVC in a cross-sectional study [42]. The authors pointed out, however, that the different carotenoids might have different effects on lung physiology.

**Table 4:** *Evidence of a relation between β-carotene and (indicators of) asthma and COPD*

| Study | Design | No. of subjects | Gender & age range (yrs) | β-carotene measured in | Outcome | Association |
|---|---|---|---|---|---|---|
| Miedema et al [16] | Prosp. | 793 | ♂ 40-60 | Diet | 25-years CNSLD | - |
| Troisi et al [17] | Prosp. | 77,866 | ♀ 34-68 | Diet | 10-years asthma | + (BS) |
| Soutar et al [22] | Cross. | 87 | ♂♀ mean 37 | Diet | $PD_{20}$ metacholine | + |
| Rautalahti et al [19] | Cross. | 29,133 | ♂ 50-69 | Diet & Serum | COPD symptoms | + |
| Morabia et al [41] | Prosp. | 83 | ♂ 50-79 | Serum | $FEV_1$ ≤75% FVC | - |
| Chuwers et al [43] | Cross. | 816 | ♂ 45-74 | Serum | $FEV_1$, FVC | + |
| Shahar et al [42] | Cross. | 10,416 | ♂♀ 45-64 | Diet (total carotene) | $FEV_1$, FVC | - |

Cross. = Cross-sectional; Prosp. = Prospective; ♀ women, ♂ men
Association: - = no association; + = beneficial association; BS = borderline statistically significant

To our knowledge studies on the relation between the intake of β-carotene and lung function have not been published before. Blood levels of β-carotene were related to lung function in two studies. A high level of serum β-carotene was not associated with the development of airway obstruction during five subsequent years [41] while serum β-carotene was positively associated with lung function in a cohort of men exposed to asbestos [43].

In conclusion, most studies that have been investigating dietary or blood vitamin C in relation to respiratory outcomes suggested a possible beneficial association in particular for lung function. No consistent pattern arises from the few studies on the relation between dietary intake or blood levels of vitamin E and β-carotene and (indicators of) asthma and COPD.

## Rationale and outline of the thesis

This thesis has two distinctive parts. The first part studies the relations between antioxidants and the acute respiratory effects of ambient air pollution in the summer and winter. The second part investigates the relations between antioxidants and indicators of asthma and COPD, such as respiratory symptoms and lung function.

### *Antioxidants in relation to the acute respiratory effects of air pollution*

In spite of the experimental evidence under controlled conditions to a single air pollutant, there is almost no evidence on a possible modulation of the acute respiratory effects of air pollution under ambient conditions with exposure to pollutant mixtures. In addition, the doses of supplementation were relatively high in these experimental studies and there have been no studies published on dietary or blood concentrations of antioxidants. So we specified the following (alternative) hypotheses:

1. The acute effect of ambient ozone on lung function is reduced by antioxidant vitamin supplementation.
2. The acute respiratory effects of winter air pollution are smaller in subjects with a high intake or a high serum level of antioxidants compared those with a respectively low intake or a low serum level of antioxidants.

The first hypothesis was investigated in two intervention studies. The results of the first intervention study are presented in *Chapter 2*. This was a pilot study during the summer of 1994 among cyclists who were supplemented with a cocktail of antioxidant (pro-) vitamins C, E and β-carotene during 10 weeks. *Chapter 3* deals with the same hypothesis as chapter 2 but this intervention study was placebo-controlled and the cyclists were supplemented with the antioxidant vitamins C and E for 15 weeks during the summer of 1996. We chose to study vitamin C and E alone in this second intervention study mainly for two reasons. First, at the start of our second intervention study the results of two large long-term supplementation studies of β-carotene on cancer incidence were published suggesting an adverse effect on lung cancer and this might have kept possible respondents from participating [44,45]. The second reason was practical namely; β-carotene was not commercially available in powder or any other substance, which was necessary for making our own capsules. *Chapter 4* focuses on the second hypothesis and combines the results of two panel (observational) studies during the winter of 1993/1994 and the winter of 1994/1995. In these studies, the investigated antioxidants were serum levels of α-tocopherol and β-carotene and the dietary intake of vitamins C, E and β-carotene.

***Antioxidants in relation to indicators of asthma and COPD***

Most of the epidemiological studies have been investigating one outcome in relation to one or two antioxidants in diet or blood. We had the opportunity to investigate the dietary intake of several antioxidants (vitamin C, vitamin E, and β-carotene) in relation to the prevalence of a number of respiratory symptoms and lung function simultaneously. In addition, we were able to study plasma antioxidants α-tocopherol and β-carotene in relation to respiratory symptoms and lung function. The following (alternative) hypotheses were specified:

1. Subjects with a high intake of antioxidants (pro)-vitamins have a lower prevalence of respiratory symptoms and a higher lung function compared to subjects with a low intake of antioxidants.
2. Subjects with a high plasma level of antioxidant (pro)-vitamins have a lower prevalence of respiratory symptoms and a higher lung function compared to subjects with a low plasma level of antioxidants.

These hypotheses were investigated in different population samples of the cross-sectional MORGEN study (the monitoring project on risk factors and health in the Netherlands). *Chapter 5* evaluated hypothesis 1 in all subjects measured in 1994 and 1995 with dietary vitamins C, E and β-carotene as exposure variables. In *Chapter 6*, the focus was on the first part of hypothesis 2; a case-control sample (subjects with and without chronic respiratory symptoms) of never and long-term former smokers was selected from the MORGEN-study within the period of July 1994 to May 1995 for determination of plasma levels of β-carotene and α-tocopherol. In *Chapter 7* a random sample from 1995 of the MORGEN-study was available with plasma levels of β-carotene and α-tocopherol for studying the relation with lung function (hypothesis 2).

Finally in *chapter 8*, the results of these studies (both part one and two of the thesis) are discussed in the context of possible biases, such as missclassification of exposure, selection bias, confounding and the choice of study design. In addition, the magnitude of the associations found in our studies will be compared with other closely related studies and the biological plausibility of the associations will be addressed.

We decided to study in this thesis the following antioxidants: vitamin C, E and β-carotene because these antioxidants are present in the lung lining fluids [46,47] suggesting that they might be able to have a beneficial effect in the lungs. In addition, these antioxidants can be altered by diet. We measured plasma concentrations of α-tocopherol and β-carotene (chapter 6 and 7), which are suggested to be valid markers for dietary intake [48], whereas plasma vitamin C is not

because of an existing homeostatic control [49,50]. In addition, plasma levels of α-tocopherol and β-carotene might be good markers for the concentration available in the lung tissue [46].

# CHAPTER 2

# Acute effects of ozone on pulmonary function of cyclists receiving antioxidant supplements: a pilot study


## Abstract

*Background* The aim of this investigation was to identify whether acute lung function effects of ozone can be modulated by antioxidant vitamin supplementation.

*Methods* Amateur cyclists (n=26) were studied in the summer of 1994 in the Netherlands. Repeated lung function measurements were performed with a rolling-seal spirometer after training session or competitive race on 4 to 14 occasions. The cyclists were assigned to two study groups. The supplementation group (n=12) received antioxidant supplements (15 mg β-carotene, 75 mg vitamin E and 650 mg vitamin C) once a day for 3 months. The control group did not receive supplementation. For each subject, post-exercise lung function was regressed on the previous 8-hour mean ozone concentration. The individual regression coefficients were pooled for each study group and weighted with the inverse of the variance.

*Results* The 8-hour mean ozone concentration was 101 $\mu g/m^3$ (30 to 205 $\mu g/m^3$). For the supplementation group, there was no effect of ozone on FVC, $FEV_1$, Peak Expiratory Flow (PEF) and Maximal Mid-Expiratory Flow (MMEF). For the control group the mean coefficients were negative, except for MMEF. The difference between the groups was 2.08 (95%CI: 1.31, 2.85) $ml/\mu g/m^3$ for FVC, 1.66 (95% CI: 0.62, 2.70) for $FEV_1$, 6.83 (95%CI: 3.17,10.49) for PEF and 0.42 (95%CI: -1.38, 2.22) for MMEF.

*Conclusion* The results suggest that antioxidant vitamin supplementation protects against acute effects of ozone on lung function in heavily exercising amateur cyclists.



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## Introduction

There is now a growing recognition that air pollution, and more specifically ozone, has adverse health effects [1,51]. Several investigators have studied pulmonary function relative to ozone and exercise. Most of these studies were conducted under controlled laboratory conditions [52-54]. After exposure to different ozone levels (160-400 $\mu g/m^3$) lung function decreased [52-54] and lower respiratory tract symptoms increased [53]. Low levels of ambient ozone on respiratory effects have been studied before in amateur cyclists. The difference between pre- and post-exercise lung function was negatively related to ozone concentrations during exercise. Shortness of breath, chest tightness and wheeze were increased after ozone exposure [55].

A possible mechanism for decrements in lung function after ozone exposure is its oxidant role, inducing (in)direct injury to lung tissues by attracting inflammatory cells. Antioxidant (pro-)vitamins, such as vitamins E, C and β-carotene, can possibly modulate the reactivity of oxidants by reacting with them before they injure the lung tissue [56]. Few laboratory studies have investigated this hypothesis, suggesting a protective role for vitamin C and a combination of vitamins C and E [10], but not for vitamin E alone [29].

As all studies were performed under laboratory conditions, it is unknown whether antioxidants can play a role in the respiratory health effects of ambient ozone in healthy adults. We explored this hypothesis in a small study measuring lung function among amateur cyclists with and without supplementation of antioxidants during summer.

## Methods

### *Study design*

Two amateur cycling clubs in Ede and Arnhem, both located in the east of the Netherlands were asked to participate in the study. From 28 June until 1 September 1994, lung function was measured in each subject before and after each training session or competitive race on a number of occasions. All training sessions and races took place in rural areas. There were no large industrial areas or cities in the surroundings. Ozone concentration data were obtained from the nearest station (Wageningen, Loenen) measured by the National Air Quality Monitoring Network operated by the National Institute of Public Health and the Environment (RIVM) in Bilthoven. Training sessions and races took place in the late afternoon and early evening when ozone levels tend to peak. The exact times were recorded for each participant in order to calculate the individual ozone exposure (8-hour mean ozone concentration before the end of exercise) and to calculate the duration of exercise.

Meteorological data were obtained from the nearest station (Deelen) operated by Royal Netherlands Meteorological Institute (KNMI) at the Bilt.

***Study population***

From 29 volunteers, 23 subjects were randomly assigned to the supplementation or control group. Five subjects were directly allocated to the control group, as they refused to take any supplementation or entered the study half way through. One subject who regularly took vitamin and/or mineral supplements and refused to stop was allocated to the supplementation group. At the beginning of the study, subjects completed a questionnaire about chronic respiratory symptoms and other relevant characteristics.

***Supplementation***

The supplementation group received 650 mg vitamin C, 75 mg vitamin E and 15 mg of β-carotene daily starting one week before the measurements started for 70 days (June-September). This supplementation was commercially available in two different pills. The control group did not receive a placebo. Before and after supplementation plasma was collected as a marker of compliance.

***Data collection***

Blood specimens were collected in ethylene diamine tetra-acetic acid (EDTA) vacutainer tubes, stored in a box on ice, and centrifuged within 5 hours to obtain plasma. Aliquots were then stored at -80 °C until analyses. Concentrations of α-tocopherol and β-carotene were measured by High Performance Liquid Chromatography (HPLC) together in one run. The column was a pre-packaged 25 cm x 4.6 mm Vydac 201TP54, $C_{18}$ 300 Å (Hesperia, CA, USA). Detection after separation was performed by two UV detectors, one for determination of retinol and carotenoids (UV2000) at wave-lengths of 325, 450 and 470 nm. The second detector (UV1000) was used for determination of the tocopherols at wave-lengths of 325 nm and 292 nm.

For compliance with exertion, continuous heart rate was measured with Polar sport testers (Polar Electro, Finland). This was measured on a number of different occasions in all subjects during trainings and races. The volumes of inhaled air were estimated with heart rates in the equations of the study of Colucci [57].

Lung function was measured before and after cycling with spirometry. All lung functions tests were conducted indoors at most 30 min. before and 10 to 60 min. after the exercise. Measurements were performed according to the ECCS guidelines [58]. The subject was seated in an upright posture, with a fixed mouthpiece adjusted

for height of every individual and without a noseclip. For each measurement, subjects had to perform at least three technically acceptable and reproducible forced manoeuvres (according to ECCS 1983 criteria), with Vicatest 5 dry rolling seal spirometers. These spirometers were coupled to a microcomputer for storage of the data. Data handling was according to the ECCS guidelines [58].The spirometers had no internal temperature sensor; however, room temperature was recorded at all times. For logistic reasons, spirometers were brought into the rooms (local clubrooms with low ceilings) shortly before the pre-exercise measurement started. We therefore had no opportunity to control indoor conditions and high temperatures (> 30 °C ) occurred regularly in these locations. The air in the spirometer bell may not have adapted to the air temperature in the clubrooms on hot days when pre-exercise tests were performed, which may have resulted in an underestimation of the pre-exercise lung function [59]. For this reason we focused the analyses on the post-exercise lung function measurements which were conducted after the spirometers had been in the clubrooms for several hours.

***Data analysis***

Before data analyses, the maximum value of three manoeuvres of each measurement was calculated for each subject for each of the following lung function variables: $FEV_1$ (forced expiratory volume in 1 second), FVC (forced vital capacity), PEF (peak expiratory flow) and MMEF (maximal mid-expiratory flow). At the end of the study, a range of four to 14 observations was available for each subject - that is, lung function measurements after exercise (post-exercise lung function). Subjects with a range in ozone exposure of less than 50 $\mu g/m^3$ (n=3) were excluded from analyses because their estimated regression coefficients would be highly unstable. Individual regression analyses were performed in each subject using SAS 'PROC REG' procedure [60] with the post-exercise lung function variable ($FEV_1$, FVC, PEF or MMEF) as the dependent variable and with the previous 8-hour mean ozone concentration as the independent variable. The resulting regression coefficients for each subject were pooled and group means (with Standard Errors), medians and means which were weighted with the inverse of the variance were calculated for the control and supplementation group. The effect of supplementation with a 95% confidence interval (95% CI) was calculated as the difference between the mean regression coefficient of the supplementation and control group. Ambient temperature, $NO_2$, and concentrations of particulate matter with a mean diameter of 10 $\mu m$ (PM10) were considered as confounding factors.

## Results

A total of 26 respondents (n=14 in control group) contributed with 192 observations to the data analyses. The average age of the respondents was 27.5 years and did not differ between the two study groups (table 1). Three participants were women. In the control group, one subject was a current smoker and two reported a doctor diagnosed pollen allergy. In the supplementation group more subjects reported having respiratory symptoms and allergy than in the control group. One subject reported having had ever doctor diagnosed attacks of chest tightness; another subject reported shortness of breath when walking at normal pace and one subject reported coughing up phlegm almost every day for three months in the last two years. Two subjects reported an allergy to pets and house dust mites, and four others an allergy to pollen, all diagnosed by a doctor.

**Table 1:** *Mean and range in age, number of observations, ozone concentration, duration of exercise,and heart rate in the two study groups*

| Measurements | Control group (n=14) | | Supplementation group (n=12) | |
|---|---|---|---|---|
| | Mean | Range | Mean | Range |
| Age* (yr.) | 28.5 | 20-41 | 27.9 | 16-39 |
| No. of observations† (n) | 6.9 | 4-14 | 7.9 | 5-12 |
| Ozone concentration‡ ($\mu g/m^3$) | 97.9 | 30-205 | 104.2 | 33-205 |
| Duration of exercise (min.) | 91.1 | 15-135 | 92.7 | 15-135 |
| Heart rate (beats/min.) | 154.0 | 132-180 | 153.1 | 129-196 |

* age of one subject from the control group is missing

† post exercise lung function measurement

‡ 8-Hour mean ozone concentration

Table 1 summarises means of number of observations, ozone concentration, duration of exercise and heart rate for the two study groups. The average 8-hour mean ozone concentration was 101 $\mu g/m^3$ (range 30-205 $\mu g/m^3$), with no difference between the two groups. The figure shows the 1-hour maximum ozone concentrations for every day from June until August. Ozone concentrations were high from the end of June (at the start of the study) until the end of July, August had lower ozone concentrations.

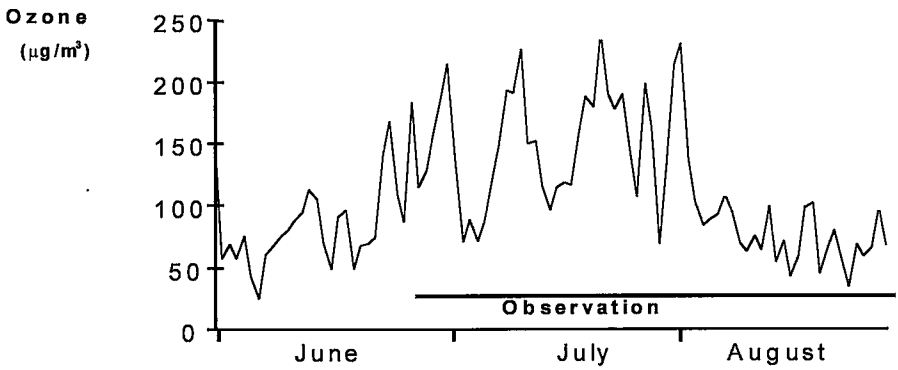


**Figure:** *Plot of the 1-hour maximum ozone concentration per day ($\mu g/m^3$) from the first of June until the first of September. Observation period was from June, 28th until September, 1st 1994*

Eight-hour mean temperature averaged 23°C, with a range of 15 to 31°C. The mean (range) duration of the exercise was not different between the two study groups (92 (15-135) minutes). Heart rate measurements were performed on 179 different occasions. During training sessions, mean (range) heart rate was 147 (129-190) bpm, and during competitive races, 165 (135-196) bpm. There was no difference between the groups. Estimated volumes of inhaled air were 55 L/min. during training and 70 L/min. during races.

**Table 2:** *α-Tocopherol and β-carotene concentrations (µmol/l) in plasma among control and supplementation group at baseline and the change during intervention*

| | Control Group (n=9) | | | | Supplementation group (n=11) | | | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | | Change* | | Baseline | | Change* | |
| | Mean | (SEM) | % | (Range) | Mean | (SEM) | % | (Range) |
| α-tocopherol | 23.0 | (7.66) | 9 | (- 7 to 21) | 30.0 | (8.35) | 38 | ( 9 to 95) |
| β-carotene | 0.32 | (0.11) | 5 | (-19 to 26) | 0.50 | (0.14) | 153 | (10 to 345) |

* Mean individual change (post minus pre divided by pre-intervention)

**Table 3:** *Post-exercise lung function variables with 8-hour mean ozone concentration by study group (control and supplementation)*

| Lung function Variable | Control (n=14) | Supplementation (n=12) | Control | Supplementation | Treatment effect |
|---|---|---|---|---|---|
| | Median* | Median* | Mean† (SEM) | Mean† (SEM) | Difference (95% CI)† |
| FVC | -0.39 | 0.44 | -1.83 (0.49) | 0.25 (0.24) | 2.08 (1.31, 2.85) |
| $FEV_1$ | -0.35 | -0.013 | -1.86 (0.49) | -0.20 (0.60) | 1.66 (0.62, 2.70) |
| PEF | -1.54 | 2.42 | -5.35 (2.03) | 1.48 (3.04) | 6.83 (3.17,10.49) |
| MMEF | -0.93 | -1.46 | -0.93 (0.94) | -0.51 (0.93) | 0.42 (-1.38, 2.22) |

* median coefficients in $ml/\mu g/m^3$

† mean coefficients (standard error of mean) in $ml/\mu g/m^3$ for FVC and $FEV_1$ and in $ml/sec/\mu g/m^3$ for PEF and MMEF weighted with inverse of variance; Difference = supplementation minus control group; 95% CI: 95% Confidence Intervals

Plasma at the beginning and at the end of the study was collected from 20 subjects (n=9 control group). The supplementation group had higher base-line concentrations of β-carotene and α-tocopherol than the control group (table 2). Individual change of β-carotene and α-tocopherol plasma concentrations were used as a marker of compliance. The supplementation group had a significantly higher increase in β-carotene and in α-tocopherol than did the control group, as expected (table 2).

Table 3 presents the median and weighted mean regression coefficients of post-exercise FVC, $FEV_1$, PEF and MMEF with 8-hour mean ozone concentration for the control and supplementation group. Median coefficients were more negative, except for MMEF, for the control group than for the supplementation group, indicating a decrease in lung function at higher ozone concentrations in the control group. After weighting the individual regression coefficients with the inverse of the variance, mean coefficients of FVC, $FEV_1$ and PEF were more negative compared to medians in the control group. The MMEF was not associated with ozone. For the supplementation group, none of the lung function parameters were associated with ozone concentrations. The weighted mean regression coefficients of FVC imply that a difference in exposure of 100 $\mu g/m^3$ ozone would decrease FVC with 183 ml in the control group and increase FVC with 25 ml in the vitamin group. The difference between the two groups was statistically significant for FVC, $FEV_1$ and PEF. There was no statistically significant difference between the study groups for MMEF.

The Pearson correlation coefficient for ozone with 8-hour mean ambient temperature was 0.93. Conventional adjustment for temperature was problematic because of this high correlation. Restricting the data to lower ambient temperatures was difficult to interpret because of the remaining few observations - that is, <40% of total observations. Adjustment for $NO_2$ as an independent variable in the model did not change the estimated regression coefficients of ozone on lung function. Neither changed the regression coefficients of ozone on lung function after adjustment for PM10.

## Discussion

The results of this study suggest that antioxidant (pro-)vitamin supplementation might protect against acute ozone effects on lung function (in particular for FVC, $FEV_1$ and PEF).

We also considered an additional regression analysis on all measurements on all subjects. After adjustment for trend, the regression coefficients of $FEV_1$ ($\beta$=-0.29 $ml/\mu g/m^3$ in the control group and $\beta$=-0.18 in the vitamin group) and FVC ($\beta$=-0.92 in the control group and $\beta$=-0.0014 in the vitamin group) were comparable to the medians of the regression coefficients presented in table 3. The difference between

the control and supplementation group was statistically significant for FVC. The unadjusted regression coefficients for PEF and MMEF were also comparable to the presented medians. So, the results were similar independent of the method of analysis, with a more negative regression coefficient in the control group compared to the supplementation group.

Adjustment of ambient temperature as a possible confounder was difficult because of the very high correlation with ozone. Ambient temperature is, however, not such an influential confounder as indicated by other studies [55,61,62]. Experimental studies showed only some potentiation of ozone effects with very high temperatures (>35 °C) and high ozone concentrations (>600 $\mu g/m^3$) [61,62]. Brunekreef and coworkers [55] also found no temperature effect under ambient conditions with an average temperature of 18 °C.

Adjustment for PM10 did not change the results. Hoek and coworkers [2] also did not find an effect of low concentrations of PM10 on the relation of ozone with lung function during a summer in The Netherlands. Neither did adjustment for $NO_2$ concentrations change the results. This could be expected since 8-hour mean $NO_2$ concentrations were low during this summer with a mean of 23 $\mu g/m^3$ and a range of 4 to 60 $\mu g/m^3$. In addition, $NO_2$ concentrations were not associated with the lung function variables (data not shown).

More subjects with respiratory symptoms and allergies were present in the supplementation group. This suggests that randomisation was not completely successful. However, the results in the present study were probably not affected because several other studies indicated that there was no difference in acute effects of ozone between groups with or without respiratory symptoms or allergies [2,51].

Baseline plasma concentrations of β-carotene and α-tocopherol were higher in the supplementation group, which could be explained by three subjects in the supplementation group who used habitual vitamin supplementation at the start of the study. Two of the subjects stopped taking extra supplementation during the study and were randomly assigned to the supplementation group. Excluding all three subjects from analyses resulted in similar regression coefficients for the supplementation group, which implies that results were not affected by these three subjects.

α-Tocopherol and β-carotene in plasma were determined in a part of the subjects to investigate group compliance. The increases in this study were comparable with other supplementation studies [63-65]. However, comparison can only be rough because duration and amount of supplementation differ between the studies. β-Carotene and α-tocopherol were combined in one capsule. Therefore, if subjects in the supplementation group had a low increase in both antioxidants this

would suggest that compliance was not complete. Although the range in individual increases in the supplementation group was large (9-95% for α-tocopherol; 10-345% for β-carotene), possibly due to individual variation of absorption of the antioxidants, there was no subject in this group who had a small increase in both antioxidants.

The control group did not receive a placebo, so the study was not blinded. It is unlikely that this could have affected the results. Both researcher and subject were not aware of the ozone concentrations at the moment of lung function measurements and therefore anticipation of the effect was not possible.

The mechanism by which ozone induces acute decrements in pulmonary function has been under discussion. Inflammatory cells increase within one hour after exposure, but reach a maximum 6 hours after exposure. This increase does not correlate well with the measured decrements in pulmonary function decrements after ozone exposure [66]. The current hypothesis for the acute decrements in lung function is a reduction of maximal inspiratory capacity through stimulation of the neural receptors in the upper airways [1,67]. These receptors are stimulated by cyclooxygenase products of arachidonic acid which are released upon ozone exposure [67]. Vitamins C and E have been shown to affect the arachidonic acid metabolism, but the role of antioxidants in this mechanism is not fully understood [10,20,68].

In summary, this study suggests that one or more of the antioxidants (vitamins C, E and β-carotene) may modulate the acute effects of ozone on lung function. Randomised placebo controlled studies with more subjects and more observations per subject are necessary to increase the precision and confirm the validity of the findings.

## Acknowledgements

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**Appendix:** Additional table for better comparison of the results with Chapter 3

*The relation[†] between 8-hour mean ozone concentration and post-exercise lung function for the overall group and by study group*

| Presentation regression coefficients | $FEV_1$ | | | FVC | | |
|---|---|---|---|---|---|---|
| | Overall group (n=26) | Control group (n=14) | Supplementation group (n=12) | Overall group (n=26) | Control group (n=14) | Supplementation group (n=12) |
| Median | -0.16 | -0.34 | -0.01 | -0.14 | -0.39 | 0.44 |
| Mean (SEM) | 0.19 (0.53) | 0.04 (0.73) | 0.37 (0.80) | -0.22 (0.49) | -0.92 (0.64) | 0.59 (0.73) |
| Mean weighted for inverse of variance (SEM) | -1.38 (0.40)* | -1.86 (0.49)* | -0.20 (0.60) | -0.71 (0.34)* | -1.83 (0.49)* | 0.25 (0.24) |
| Mean weighted for number of measurements (SEM) | 0.01 (0.45) | 0.02 (0.61) | 0.01 (0.69) | -0.09 (0.42) | -0.49 (0.56) | 0.31 (0.65) |

* $p<0.05$

† presented as regression coefficients (ml/μg/m$^3$)

# CHAPTER 3

# A double-blind intervention trial on modulation by antioxidant supplements of ozone effects on pulmonary function


## Abstract

*Background* The aim of this study was to investigate whether acute effects of ozone on lung function can be modulated by antioxidant vitamin supplementation in a placebo-controlled study.

*Methods* Lung function was measured in Dutch cyclists (n=38) before and after each training on a number of occasions (n=380) during the summer of 1996. The vitamin group (n=20) received daily 100 mg vitamin E and 500 mg vitamin C for 15 weeks.

*Results* Average ozone concentration during exercise was 77 $\mu g/m^3$ (range: 14-186 $\mu g/m^3$). After excluding subjects with insufficient compliance from the analysis, a difference in ozone exposure of 100 $\mu g/m^3$ would decrease forced expiratory volume in 1 second with 95 ml (95% confidence interval (95% CI):-265 to –53 ml) in the placebo group and with 1 ml (95%CI –94 to 132 ml) in the vitamin group; for forced vital capacity this was -125 ml (95% CI –384 to –36) in the placebo group and -42 ml (95% CI –130 to 35 ml) in the vitamin group. The differences in ozone effect on lung function between the groups were statistically significant.

*Conclusions* The results suggest that supplementation of antioxidant vitamins C and E confers partial protection against acute effects of ozone on $FEV_1$ and FVC in cyclists.


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## Introduction

Acute effects of ozone on lung function have been studied in exercising subjects. Most of these studies were conducted under controlled laboratory conditions [52-54,69-72]. All of these chamber studies showed that lung function decreased after exposure to high levels of ozone (160-700 $\mu g/m^3$). However, low levels of ambient ozone (< 120 $\mu g/m^3$) were also negatively related to lung function [55] in exercising cyclists.
In addition to lung function decrements, which occur within one hour after acute ozone exposure, an increase in inflammatory mediators was shown in bronchoalveolar lavage within one hour [73,74], six hours [66], 18 hours [73,74], and 24 hours [66] of exposure. This increase of inflammatory mediators was different in time for each mediator [66,73,74]. Antioxidants, such as vitamin C and E, could modulate the airway response to ozone by reducing attraction of inflammatory cells [56]. Since lung function decrements after ozone exposure occur earlier in time compared to the increase of inflammatory cells, another hypothesis could explain the modulation of ozone effects on lung function: maximal inspiratory capacity is reduced through stimulation of the neural receptors in the upper airways by cyclooxygenase products of arachidonic acid which are released upon ozone exposure [67]; vitamins C and E have been shown to affect this arachidonic acid metabolism, but the role of these vitamins in this mechanism is not fully understood [10,20,68].

Few experimental studies have investigated a possible protective role of antioxidants on acute effects of ozone. One study showed that a supplementation of vitamin C and a combination of vitamin C and E protected against decrements in lung function in subjects exposed to ozone concentrations of 600 $\mu g/m^3$ [10]. Another study did not show a protective role of vitamin E alone on lung function after exposure to 1000 $\mu g/m^3$ ozone [29].

We explored a possible modulation of antioxidants on acute effects of ambient ozone in a placebo-controlled study, supplementing cyclists with antioxidant vitamins C and E and measuring lung function on a number of occasions in the Netherlands during the summer of 1996.

## Methods

### *Study design and population*

Two cycling clubs in Apeldoorn and Nijmegen, which are located in the east of the Netherlands, were asked to participate in the study. From these clubs both amateur and recreational cyclists participated. A recreational club in Malden, also located in the east of the Netherlands, volunteered to participate after an advertisement for new subjects. From these three clubs, 46 non-smoking subjects volunteered and were randomly assigned to the placebo or vitamin group. An earlier study in 1991 [55] suggested that 23 cyclists with on average 12 measurements per cyclist was able to

show an effect of ozone with relatively low levels of ozone. So the number of subjects in our study (n=46) would allow us to detect an effect of ozone in the control group or the absence of an effect in the vitamin group. From 20 May until the end of August 1996, lung function was measured before and after each training session or competitive race on a number of occasions in each subject. Most training sessions and competitive races took place in rural areas in the late afternoon and early evening, when ozone levels tend to peak. The exact times of each exercise period were recorded for each participant in order to calculate the individual ozone exposure (mean ozone concentration during exercise and 8-hour mean ozone concentration before post-exercise lung function measurement) and to calculate the duration of exercise. Daily concentrations of both ozone and particulate matter with a 50 percent cut-off diameter of 10 μm (PM10) were obtained from the nearest monitoring stations (Loenen, Wageningen) of the National Air Quality Monitoring Network which is operated by the National Institute of Public Health and the Environment (RIVM) at Bilthoven. Meteorological data were obtained from the nearest station (Deelen) operated by the Royal Netherlands Meteorological Institute (KNMI). Airborne pollen concentrations were obtained from the department of Lung Diseases, Academic Hospital in Leiden. At the beginning of the study, subjects completed a questionnaire about chronic respiratory symptoms and other relevant characteristics.

***Supplementation***

The vitamin group (n=19) received a daily dose of 500 mg vitamin C and 100 mg vitamin E in two capsules starting one week before the measurements for a total duration of 15 weeks (May-August). Subjects stopped taking their own vitamin supplements (three subjects used daily multivitamins, vitamin C or vitamin E) at least one week before the start of the study. The vitamin capsules and the placebos were hand made, visually identical and given each a code which was not known to the involved researchers and subjects. At the end of the study, the subjects returned the remainder of the capsules (a supply for 20 weeks was given) so we could estimate compliance. Next to counting the capsules, an additional measure of compliance was evaluated; subjects were asked at the end of the study if they had not been taking the capsules for longer than a week consecutively. Plasma was collected before and at the end of supplementation for determination of α-tocopherol and vitamin C as additional markers of compliance.

***Data collection***

Blood specimens were collected in ethylene diamine tetra-acetic acid (EDTA) vacutainer tubes, stored in a box on ice, and centrifuged within five hours to obtain

plasma. Aliquots were then stored at -80 °C until analyses. Concentrations of α-tocopherol at base-line and after supplementation in each subject were measured by reverse-phase high-performance liquid chromatography (HPLC) together in one run. The method was adapted from Hess and coworkers [75]. The column was a prepackaged 25 cm x 4.6 mm Vydac 201TP54, $C_{18}$ 300 Å (Hesperia, CA, USA). The mobile phase consisted of methanol-tetrahydrofuran-water in the following configuration (% v/v/v): 0 min. 89:2:9; 10 min. 98:2:0; 20 min. 97:3:0; 30 min. 90:10:0; 40 min. 90:10:0 with a flow rate of one ml/min. Detection after separation was executed using an ultraviolet (UV) detector (UV1000) for determination of the tocopherols at wave lengths of 325 nm for 0-9 minutes and 292 nm for 9-40 minutes. Plasma vitamin C was analyzed by a modification of the method developed by Roe and Kuether [76] at base-line and at the end of the intervention period in these subjects.

Continuous heart rate measurements were performed as a measure of exertion, using Polar sporttesters (Polar Electro, Finland) on a number of different occasions in all subjects during trainings and competitive races. With these mean heart rates during exercise, the volumes of inhaled air were estimated according to the equation from the study of Colucci [57] for subjects with an age range of 21 to 60 years.

Lung function was measured before and after cycling with a heated pneumotachometer (Jaeger, Germany). The flow-volume and the volume-time curves were observed on the attached computer for all maneuvers during the measurement; all data was automatically saved on the computer. Temperature at location was measured and controlled for during the measurements by entering the temperature into the computer. Next to the calibration with a one liter syringe, the researchers measured their own lung function twice at the same time as the cyclists, to check for abnormalities of the equipment. All lung functions tests were conducted indoors, at most 60 minutes before and on average 17 minutes (range: 5-60 min.) after the exercise. Pre- and post-exercise lung function was measured on the same pneumotachometer. Measurements were performed according to the European Respiratory Society (ERS) guidelines [77]; subjects were seated in an upright posture, with a fixed mouthpiece adjusted for height of every individual and a nose clip was used. For each measurement, subjects had to perform at least three technically acceptable of which two reproducible (according to ERS 1993 criteria) forced maneuvers.

***Data analysis***

In data analysis, maximum values of the three maneuvers of each measurement were used in each subject of the following lung function parameters: Forced

Expiratory Volume in 1 second ($FEV_1$), Forced Vital Capacity (FVC), Peak Expiratory Flow (PEF) and Maximal Mid-Expiratory Flow (MMEF). Before data analysis, two criteria were set for subjects to be included in the individual regression analysis to prevent the occurrence of unstable regression coefficients. First subject had to have at least four observations, that is, lung function measurements before and after exercise (pre- and post-exercise lung function respectively). Second each subject should have a range in ozone or PM10 exposure of more than 40 $\mu g/m^3$. All subjects fulfilled this second criterion for ozone exposure during exercise and for 8-hour mean ozone exposure. Six subjects had a range of PM10 exposure of less than 40 $\mu g/m^3$ and were excluded from the analysis of PM10 concentrations in relation to lung function. PM10 concentrations were calculated as 24-hour mean concentrations previous to the post-exercise lung function measurement. In addition, a lag of one and two days was calculated. Twenty-four hour mean PM10 concentrations were missing for three days but all subjects had still at least four observations for the analysis of PM10 in relation to lung function.

Data were analyzed using Statistical Analysis System (SAS) procedures. For each subject, delta lung function (post minus pre-exercise lung function) as dependent variable was regressed on the ozone concentration (in $\mu g/m^3$; 1 $\mu g/m^3$ of ozone equals to 0.5 ppb) during exercise. We also considered for each subject post-exercise lung function measurements as the dependent variable with the previous 8-hour mean ozone concentration as the independent variable because exposure to ozone prior to the exercise could have affected the pre-exercise lung function levels. The resulting individual regression coefficients were pooled in both analyses and medians and means (with standard errors of the means) were calculated for the total group and for the control (placebo) and vitamin group separately. In addition, weighted group means were calculated with the inverse of the variance of the individual regression coefficient as weight. The 95% confidence intervals (95% CI) around the median regression coefficients were calculated with a non-parametric method published by Campbell and Gardner [78]. The difference between the vitamin and control group was tested for statistical significance ($p<0.05$) with the Wilcoxon rank-sum test.

The following potential confounders were taken into account in the regression analyses: ambient temperature, absolute humidity, PM10 concentrations with a lag of zero, one, two days and an interaction term of ozone and PM10 (a dummy for PM10 with a cut-off point at the median times the ozone concentration) and pollen concentrations (Poaceae [grass] and Betula [birch]). Pearson correlation coefficients were calculated between the independent variables. There were too few subjects with self-reported asthma (n=4) and/or inhalant allergies (n=8) to analyze this subgroup separately. Therefore, we examined if the regression coefficients were

affected when excluding asthmatic or allergic subjects from analyses. The effect of insufficient compliance was considered by excluding subjects who returned too few or too many capsules (equivalent to more than 110 percent or less than 80 percent of the planned cumulative dose respectively). In addition, subjects who reported that they had not been taking the capsules for three consecutive weeks or more were excluded.

## Results

Of the 46 subjects at the beginning of the study, eight subjects were excluded from analyses; five subjects had less than four measurements, one subject dropped out immediately because he could not swallow the capsules and two subjects were not able to perform reproducible lung function measurements.

The 38 remaining subjects had a total of 380 lung function measurements (i.e. pre- and post-exercise lung function measurements) with a range of 5-19 measurements per subject. Three subjects were female with one female subject in the control group. Eight subjects reported a doctor diagnosed allergy to pets, house dust mite or pollen; four subjects reported doctor diagnosed asthma. A slightly higher number of these allergic (n=5) and asthmatic subjects (n=3) were allocated to the vitamin group compared to respectively three allergic and one asthmatic subject(s) in the control group. Respiratory symptoms and the use of vitamin supplements before the start of the study were not different between the groups.

Table 1 summarizes the mean age, baseline $FEV_1$ and FVC, number of measurements, compliance by counting the number of the returned capsules, ozone concentration during exercise and 8-hour mean ozone concentration. There was no difference in means or medians (results not shown) between the groups, except for compliance: the control group showed a somewhat higher compliance (97 vs. 86 percent). Of these 38 subjects, 33 had data on plasma $\alpha$-tocopherol before and after supplementation. Mean baseline plasma $\alpha$-tocopherol was 27.2 (standard error 1.17) mmol/l and plasma vitamin C was 81.2 (standard error 2.9) $\mu$mol/l which were both not different between the study groups. After supplementation, the mean of the individual changes for plasma $\alpha$-tocopherol was 3.8 percent (range of -12 to 19 percent) in the control group (n=15) and 48.4 percent (range of -2 to 134 percent) in the vitamin group (n=18). For plasma vitamin C the mean individual changes were –4.3 percent (range –58.1 to 37.3) in the control group and 4.1 percent (range –18 to 49 percent) in the vitamin group.

**Table 1:** *Mean and range in age, number of observations, compliance and ozone concentrations in the two study groups in 38 Dutch cyclists, 1996*

| Characteristics, measurements | Control group (n=18) | | Vitamin group (n=20) | |
|---|---|---|---|---|
| | Mean | Range | Mean | Range |
| Age (yr.) | 33.1 | 17-58 | 33.8 | 16-59 |
| Baseline lung function | | | | |
| $FEV_1$ (L) | 4.7 | 3.6-6.0 | 4.6 | 2.7-7.2 |
| FVC (L) | 5.9 | 4.2-7.0 | 5.8 | 3.9-8.0 |
| No. of observations | 10.2 | 5-19 | 9.9 | 5-17 |
| Compliance (%) † | 96.8 | 70-130 | 86.1 | 53-101 |
| Ozone concentration during exercise ($\mu g/m^3$) ‡ | 75.4 | 17-181 | 78.4 | 14-186 |
| Eight-hour mean ozone concentration ($\mu g/m^3$) | 84.2 | 32-199 | 88.2 | 33-199 |

$FEV_1$: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity

† Compliance for subject expressed as % of the number of capsules which they should have been taken

‡ 1 $\mu g/m^3$ equals to 0.5 ppb

Figure 1 shows the 1-hour maximum ozone concentrations for every day from May until August 1996.

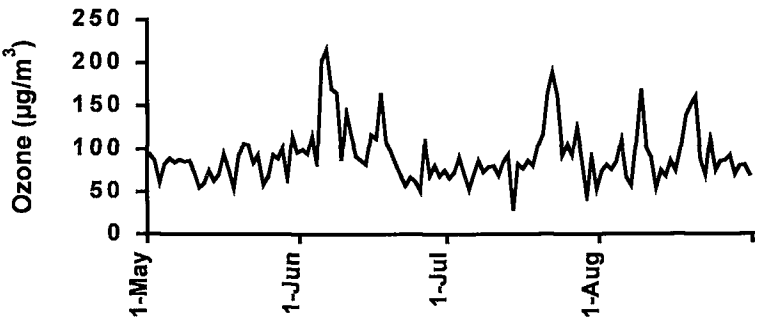


**Figure 1:** *Plot of the 1-hour maximum ozone concentration per day ($\mu g/m^3$) from the first of May until the end of August. Observation period was from May, 20th until August, 27th 1996*

The average ozone concentration during exercise was 77 $\mu g/m^3$. Mean temperature during exercise was 17 °C, with a range of 10 to 25 °C. Eight-hour mean temperature was 19 °C with a range from 12 to 28 °C. Mean absolute humidity was 13 mg/l air with a range of 9 to 20 mg/l air. The average 24-hour mean PM10 concentration was 41 $\mu g/m^3$ with a range of 13 to 144 $\mu g/m^3$. Pearson correlation coefficients of 8-hour mean ozone concentration with 8-hour mean temperature was 0.59, with 8-hour mean absolute humidity 0.28, with 24-hour mean PM10

concentrations 0.37. The mean duration of exercise was not different between the two groups with a mean of 104 min. (range: 20-216 min.). Heart rate measurements were performed on 315 different occasions with an average for training sessions of 141 bpm (range: 83-182 bpm) and for competitive races of 173 bpm (range: 156-187 bpm). There was again no difference between the two groups. Mean estimated volumes of inhaled air were 55 L/min. during trainings and 92 L/min during races [57].

Table 2 compares the ozone effects in the vitamin group versus the control group for both delta lung function and post-exercise lung function as outcome variables. There was a significant negative effect of ozone on delta-$FEV_1$ and delta-FVC but not on delta-PEF and delta-MMEF. Ozone exposure was also negatively associated with post-exercise $FEV_1$, FVC and PEF. Further analyses were concentrated on $FEV_1$ and FVC which were more reproducibly measured and showed similar magnitudes of the regression coefficients for both delta lung function and for post-exercise lung function compared to PEF and MMEF. Because pre-exercise lung function could have been influenced to an unknown extent by ozone exposure prior to the trainings or races, we decided to focus on post-exercise lung function and on 8-hour average exposures as well. The median regression coefficients of 8-hour ozone on post-exercise $FEV_1$ and FVC were significantly different from zero in the control group, whereas the regression coefficients were not different in the vitamin group. The differences in the effect of ozone between the study groups were not statistically significant (table 2).

Table 3 compares the effect of 8-hour mean ozone on post-exercise $FEV_1$ and FVC between the control and vitamin group after adjustment for 24-hour mean PM10, 8-hour mean temperature and compliance. After adjustment for PM10 and temperature, the differences in ozone effects on lung function between the study groups generally became somewhat larger compared to the unadjusted differences (table 2). After excluding insufficient compliers, ozone exposure was significantly associated with $FEV_1$ and FVC in the control group whereas ozone was not associated with lung function in the vitamin group. The differences in ozone effect on lung function between the groups were statistically significant (table 3).

**Table 2:** *Median regression coefficients and 95% confidence intervals (95% CI) of ozone on delta lung function and post-exercise lung function in the total group of 38 Dutch cyclists and in the control group (n=18) and vitamin group (n=20) separately, 1996*

| Lung function parameter | Dependent variable | | | | | |
|---|---|---|---|---|---|---|
| | Delta lung function† | | | Post-exercise lung function‡ | | |
| | Total group | Study group | | Total group | Study group | |
| | | Control group | Vitamin group | | Control group | Vitamin group |
| | Median (95% CI) | Median (95% CI) | Median (95% CI) | Median (95% CI) | Median (95% CI) | Median (95% CI) |
| $FEV_1$ | -0.99 (-1.89, -0.32) | -1.19 (-2.34, -0.39) | -0.68 (-2.76, 0.71) | -0.67 (-0.96, -0.44) | -0.95 (-2.60, -0.62) | -0.50 (-0.95, 0.52) |
| FVC | -1.03 (-1.24, -0.24) | -1.03 (-2.99, -0.05) | -1.10 (-2.24, -0.09) | -1.05 (-1.30, -0.37) | -1.18 (-2.78, -0.74) | -0.61 (-1.84, 0.31) |
| PEF | 1.14 (-4.23, 4.24) | 1.55 (-5.67, 5.36) | 1.14 (-8.05, 8.07) | -4.01 (-6.08, -2.90) | -3.80 (-6.64, -1.64) | -4.36 (-7.29, -1.87) |
| MMEF | -1.11 (-3.42, 0.16) | -2.86 (-4.43, 0.82) | -0.43 (-3.42, 3.14) | -0.47 (-1.69, 1.33) | -0.28 (-2.99, 1.33) | -0.88 (-2.07, 2.72) |

$FEV_1$, Forced Expiratory Volume in one second; FVC, Forced Vital Capacity; PEF, Peak Expiratory Flow; MMEF, Maximal Mid-Expiratory Flow

† Median coefficients of post-exercise minus pre-exercise lung function on ozone during exercise in ml per $\mu g/m^3$ ozone for $FEV_1$ and FVC and in ml/sec per $\mu g/m^3$ ozone for PEF and MMEF

‡ Median coefficients of post-exercise lung function on 8-hour mean ozone in ml per $\mu g/m^3$ ozone for $FEV_1$ and FVC and in ml/sec per $\mu g/m^3$ ozone for PEF and MMEF

**Table 3:** *Median regression coefficients (in ml per μg/m³ ozone) and 95% confidence intervals (95% CI) of post-exercise $FEV_1$ and FVC on 8-hour mean ozone by study group after different adjustments in Dutch cyclists, 1996*

| **Adjustments/ exclusion** | Total group | Study group | |
|---|---|---|---|
| Lung function parameter | (n=38) | Control (n=18) | Vitamin (n=20) |
| | Median (95% CI) | Median (95% CI) | Median (95% CI) |
| **24-Hour mean PM10** | | | |
| $FEV_1$ | -0.71 (-1.64, -0.35) | -1.36 (-2.80, -0.35) | -0.65 (-1.64, 1.20) |
| FVC | -1.33 (-2.06, -0.30) | -1.89 (-2.75, -0.82) | -0.11 (-1.93, 0.74) † |
| **8-Hour mean temperature** | | | |
| $FEV_1$ | -1.25 (-1.94, -0.20) | -1.58 (-2.63, -0.44) | -0.08 (-3.21, 2.06) |
| FVC | -1.69 (-2.66, -0.46) | -1.89 (-3.03, -1.31) | -0.37 (-4.41, 0.97) |
| **Excluding insufficient compliers** ‡, unadjusted | (n=28) | (n=14) | (n=14) |
| $FEV_1$ | -0.67 (-0.95, 0.24) | -0.95 (-2.65, -0.53) | -0.01 (-0.94, 1.32) † |
| FVC | -1.00 (-1.30, -0.36) | -1.25 (-3.84, -0.36) | -0.42 (-1.30, 0.35) † |

† vitamin group differs significantly from control group ($p < 0.05$, Wilcoxon rank-sum test)

‡ see text in methods

The crude median regression coefficients after exclusion of insufficient compliers imply that a difference in exposure of 100 μg/m³ ozone would decrease $FEV_1$ by 95 ml and FVC by 125 ml in the control group and decrease $FEV_1$ by 1 ml and FVC by 42 ml in the vitamin group (table 3). After adjustment for PM10 and temperature, the median regression coefficients of ozone on lung function of good compliers decreased slightly in the control group and increased slightly in the vitamin group increasing so the differences between the groups. The unweighted and weighted mean regression coefficients of ozone on lung function in both study groups showed similar patterns in the results (results not shown).

Adjustment for pollen concentrations or absolute humidity did not materially change the estimated regression coefficients. This was also true when excluding asthmatics or allergic subjects (results not shown). PM10 with a lag of zero, one and two days were not associated with post-exercise $FEV_1$ and FVC. An interaction term of PM10 and ozone was not different from zero (results not shown).

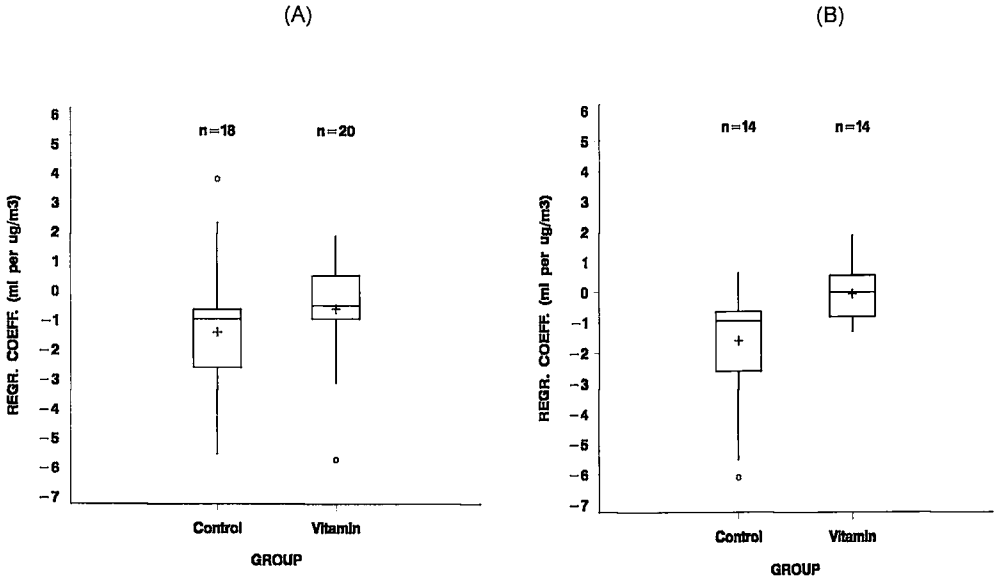


**Figure 2:** *Boxplot of the distribution of regression coefficients of $FEV_1$ in the control group and vitamin group with median (center of the box), mean (plus sign), 25th and 75th percentile (borders of the box, i.e. interquartile range), range (whiskers) and outliers; (A) all subjects, (B) good compliers only*

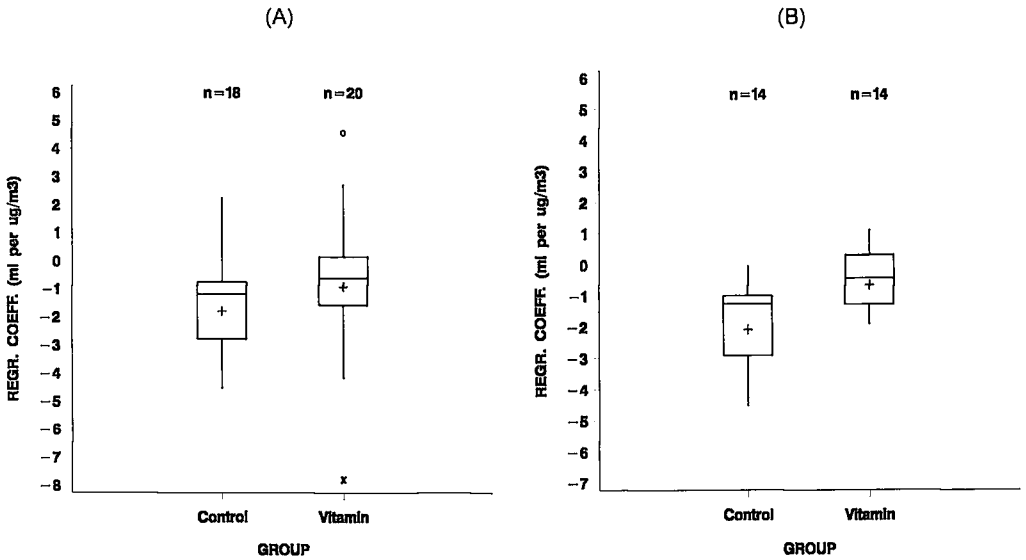


**Figure 3:** *Boxplot of the distribution of regression coefficients of FVC in the control group and vitamin group with median (center of the box), mean (plus sign), 25th and 75th percentile (borders of the box, i.e. interquartile range), range (whiskers) and outliers; (A) all subjects, (B) good compliers only*

Figure 2 shows the distribution of the crude regression coefficients of 8-hour mean ozone on post-exercise $FEV_1$ for all subjects (figure 2A) and in good compliers (figure 2B). Figure 3 shows the same distributions for FVC in all subjects (figure 3A) and in good compliers (figure 3B). These figures illustrate that the distribution of regression coefficients of ozone on $FEV_1$ and FVC was shifted upwards in the vitamin group compared to the control group and that this became more clear after excluding the insufficient compliers.

## Discussion

The results of this study show that relatively low concentrations of ozone were associated with a decrease in post-exercise $FEV_1$ and FVC. The effect was stronger in the control group receiving placebo treatment suggesting that supplementation of vitamins C and E was able to partially remove acute effects of ozone on lung function in heavily exercising subjects.

Since the subjects were cycling in ambient air and ozone concentrations tend to be uniform over large areas in the Netherlands, we assumed that the level of ozone at the monitoring site was a good proxy for the level of ozone at the place they were cycling. The monitoring sites in our study were less than 50 km from the training or racing sites and a correlation coefficient of 0.82 was observed at a distance of more than 100 km between two monitoring sites in the Netherlands [2]. It is known that ozone concentrations are lower in urban areas but as our monitoring site was in a non-urban location and cycling took place in non-urban areas as well, there is no reason to believe that the level of ozone exposure of our cyclists was different from the measurements of the monitoring network site.

Adjustment for ambient temperature and absolute humidity did not change the results considerably. Experimental studies showed only some potentiation of ozone effects with very high temperatures (> 35 °C) and high ozone concentrations (> 600 $\mu g/m^3$) [61,62]. Previously, we did not find an effect of temperature or absolute humidity under ambient conditions with an average temperature of 18 °C which was very comparable to the present study [55].

Adjustment for PM10 did not essentially change the results. Hoek and coworkers [2] did also not find an effect of low concentrations of PM10 on the relation between ozone and lung function during a summer in the Netherlands.

The effect of insufficient compliance (number of pills taken) was investigated. There was no change in the control group which is understandable since an intake of fewer placebos is unlikely to change the results. There were, however, no longer

effects of ozone on lung function in the vitamin group when insufficient compliers were excluded.

Differences in plasma concentrations of α-tocopherol and vitamin C between base-line levels and the end of the study were taken as markers for group compliance. However, plasma vitamin C was not a good marker in this study because these subjects were young and healthy and their base-line levels of vitamin C were already in the plateau phase (68-85 μmol/l) of the intake [34]. So, a higher supplemental intake would not lead to higher plasma levels in this group of subjects [50]. Plasma level of α-tocopherol is considered to be a relatively good marker of the intake of vitamin E because they are moderately reactive to vitamin E intake [48] although the absorption of vitamin E may be incomplete (20-80%) [34] and variable because the absorption declines with increasing dose of vitamin E intake [79]. Our results showed that there was a clear increase in plasma levels of α-tocopherol in the vitamin group whereas this was not so in the placebo group. This was similar to our findings in a pilot study conducted in 1994 with a supplementation of 75 mg vitamin E and an increase of nine percent and 38 percent in plasma α-tocopherol in the control and vitamin group respectively [80]. The increase of plasma α-tocopherol in this study was also comparable with other supplementation studies [64,65], although comparison can only be crude because the length and amount of supplementation differ between the studies.

Chatham and coworkers [10] found that a daily supplementation of vitamin C (1 g just before ozone exposure) and vitamin E (800 mg daily) protected partly to acute effects of ozone (600 $\mu g/m^3$) on $FEV_1$ and FVC in 14 healthy adults. Preliminary results of a recent experimental study suggested that peak expiratory flow decrements from $SO_2$ (0.10 & 0.25 ppm) challenges for two times ten minutes after 45 minutes of ozone exposure (240 $\mu g/m^3$) were significantly less during the vitamin regimen (500 mg vitamin C and 400 mg vitamin E) compared to the placebo regimen in exercising subjects (n=6) with severe asthma [32]. Recently, an experimental study [33] supplemented healthy non-smoking subjects daily with vitamin C (250 mg), α-tocopherol (100 mg) and vegetable cocktail. After two weeks, the subjects were exposed to 800 $\mu g/m^3$ (0.4 ppm) for two hours with moderate exercise. Preliminary results suggest that the lung function decrements were lower in the antioxidant group compared to the placebo group. However, there was no change in the inflammatory endpoints between the groups [33]. In the summer of 1994, we performed a pilot study with a similar design as in the present study among cyclists (n=26) in the Netherlands. Half of the subjects were supplemented with a daily cocktail of vitamin C (650 mg), vitamin E (75 mg), and beta-carotene (15 mg) for 3 months; the control

group did not receive a placebo. Post-exercise lung function was related to 8-hour mean ozone concentrations which were slightly higher (101 $\mu g/m^3$) compared to this study; the weighted mean regression coefficients were more negative in the control group and less negative in the vitamin group compared to the present study [80]. Street workers (n=49) in Mexico-city received a similar cocktail as the cyclists of 1994 in a placebo-controlled study [31]. Although the street workers were not exercising heavily, the mean one-hour maximum ozone concentration was higher (110 ppb, equivalent to 163 $\mu g/m^3$ for conditions in Mexico City) and the results suggest a beneficial effect of the antioxidants on $FEV_1$, FVC and MMEF.

In the present study, a supplementation of vitamins C and E might protect a decrement in lung function of about two percent for $FEV_1$ and 1.5 percent for FVC if there was a difference in exposure to ozone of 100 $\mu g/m^3$ in the group of good compliers. This percentage of protection is about equal to the effect of ozone in the control group and might be classified as a mild response to ozone, in particular, in this group of healthy subjects [1].

In summary, the present study suggests that supplementation of the antioxidant vitamins C and E protects partly against the acute effects of low levels of ozone on $FEV_1$ and FVC in cyclists.

## Acknowledgments

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**Appendix:** Additional table for better comparison of the results with Chapter 2

*The relation[†] between 8-hour mean ozone concentration and post-exercise lung function for the overall group and by study group*

| Presentation regression coefficients | $FEV_1$ | | | FVC | | |
|---|---|---|---|---|---|---|
| | Overall group (n=38) | Control group (n=18) | Vitamin group (n=20) | Overall group (n=38) | Control group (n=18) | Vitamin group (n=20) |
| Median | -0.67* | -0.95* | -0.50 | -1.05* | -1.18* | -0.61* |
| Mean (SEM) | -0.83 (0.29)* | -1.26 (0.46)* | -0.46 (0.35) | -1.17 (0.31)* | -1.63 (0.40)* | -0.76 (0.46) |
| Mean weighted for inverse of variance (SEM) | -0.46 (0.20)* | -0.77 (0.31)* | -0.09 (0.25) | -0.67 (0.15)* | -1.36 (0.25)* | -0.40 (0.17)* |
| Mean weighted for number of measurements (SEM) | -0.92 (0.28)* | -1.30 (0.42)* | -0.56 (0.35) | -1.24 (0.30)* | -1.61 (0.37)* | -0.89 (0.46) |

* $p<0.05$

† presented as regression coefficients ($ml/\mu g/m^3$)

# CHAPTER 4

# Modulation of the acute respiratory effects of winter air pollution by serum and dietary antioxidants: a panel study


## Abstract

*Background* We investigated whether a high dietary intake or serum concentration of antioxidant (pro-) vitamins could attenuate the acute respiratory effects of air pollution in panels of 50-70 year old adults (n=227) with chronic respiratory symptoms in the winters of 1993/1994 and 1994/1995.

*Methods* Subjects performed daily peak expiratory flow (PEF) measurements in the morning and evening and reported the occurrence of respiratory symptoms in two regions (urban and non-urban) each winter. Logistic regression analysis was used with the prevalences of large PEF decrements (more than 10% or 20% below the subject-specific median) or respiratory symptoms as dependent variables. Analyses were performed separately for subjects below and above the median levels of dietary vitamin C and β-carotene and serum β-carotene.

*Results* Subjects with low levels of serum β-carotene (<0.30 μmol/l) had more often large PEF decrements when particles <10 μm in diameter or black smoke levels were high compared to subjects with high levels of serum β-carotene. No relation was found with dietary vitamin C or β-carotene.

*Conclusions* The results suggest that serum β-carotene was able to attenuate respiratory effects of air pollution in a panel with chronic respiratory symptoms. Dietary vitamin C and β-carotene did not modify the acute respiratory effects of winter air pollution.


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## Introduction

The acute effects of air pollution on peak expiratory flow (PEF) and respiratory symptoms have been investigated in several panel studies. Some of these studies were performed on children [3,5,6] and others on adults with chronic respiratory symptoms [4,81,82]. The findings of these studies are consistent with an adverse effect of particular matter with an aerodynamic diameter less than 10 μm (PM10) and ozone on lung function and respiratory symptoms.

A possible beneficial effect of antioxidant supplements has been suggested in two small experiments investigating the acute effects of winter air pollution on respiratory health in policemen directing traffic in a city during the winter (the concentrations of air pollutants were unknown) [11]. A single supplement of vitamin C prevented a decrease in lung function ($MEF_{50}$) and an increase in airway responsiveness to histamine after two hours of traffic directing. The other experiment suggested that a vitamin C supplement for four consecutive days on each morning before work attenuated a decrease in PEF during the workday that was presumably due to air pollution [11]. Modulation of the acute effects of $NO_2$ by supplemental antioxidants was observed in two experimental studies. Three hour of exposure to 7,520 $\mu g/m^3$ $NO_2$ showed lower levels of lipid peroxidation products in the lung lavage fluid in the group supplemented with vitamins C and E [30]. One hour of exposure to 3,760 $\mu g/m^3$ $NO_2$ showed a reduction of airway responsiveness to metacholine in the group with vitamin C supplements [12]. Most of the evidence of a possible beneficial effect of antioxidants comes, however, from studies investigating ozone in relation to lung function [10,31,32,80,83]. All of these studies evaluated the effect of extra supplemental antioxidants above the normal dietary intake.

Within the framework of a larger panel study on the acute effects of winter air pollution on respiratory health of 50-70 years old adults (results will be published elsewhere), we investigated whether dietary or serum antioxidant (pro)-vitamins were associated with the acute respiratory effects of winter air pollution. In this panel study, acute effects of winter air pollution were only observed in the panels of adults with chronic respiratory symptoms and we restricted our investigation on antioxidants to these panels.

## Methods

### *Study design*

The present study was designed within the framework of a large investigation on the relation between exposure to winter air pollution (3 subsequent winters) and acute respiratory health in selected panels of children and adults with and without chronic respiratory symptoms in urban and non-urban control areas in the Netherlands. The

results of this investigation will be published elsewhere. The data for the present study were collected during two consecutive winters from November to February starting in 1993/1994 with four panels during each winter: adults with and without chronic respiratory symptoms from an urban area and a non-urban control area. As mentioned, the current investigation of modulation by antioxidants is restricted to the panels of adults with chronic respiratory symptoms because we found only in these panels acute respiratory effects of air pollution. The urban area in both winters was Amsterdam, the Netherlands with about 720,000 inhabitants. The control areas (Meppel in 93/94, Nunspeet in 94/95) were small towns with about 25,000 to 32,000 inhabitants. The participants performed daily measurements of peak flow (PEF) and completed symptom diaries. Air pollution was monitored daily at fixed sites in each area. At the beginning of the study period, subjects underwent a medical characterisation which included measurements of lung function, bronchial responsiveness, allergen skin prick testing and blood sampling for determination of the concentrations of serum total IgE and the antioxidants α-tocopherol and β-carotene. In addition, participants filled out a semi-quantitative food frequency questionnaire and a general questionnaire. This general questionnaire provided additional information about chronic respiratory symptoms, medication use, family history of asthma and allergies, occupation, demographic, life-style and environmental factors.

***Study population***

Subjects were approached by mail and invited to participate in the study. Names and addresses from subjects, aged 50-70 years, were obtained by a random sample of the municipal registration. Subjects with chronic respiratory symptoms in each area were selected with a screening questionnaire, which consisted of selected questions of chronic respiratory symptoms (chronic cough, chronic phlegm, productive cough, wheeze with shortness of breath and without having a cold or flu, shortness of breath) from the Dutch part of the European Community Respiratory Health Survey [84]. In the second winter, two exclusion criteria were added: use of beta-blockers (contra-indication for bronchoprovocation test) and work outside of the hometown. Only subjects who signed an informed consent were included in the study. In the first winter, the planned panel size of subjects with chronic respiratory symptoms was 75 subjects in each of the urban and non-urban control area resulting in a total of 150 subjects. In the second winter, the planned panel size was 60 subjects in the panel with chronic respiratory symptoms resulting in a total of 120 subjects for the analysis.

***Data collection***

During the study period, peak flow measurements were performed twice a day before breakfast in the morning and before going to bed for three months using Mini Wright peak flow meters. The participants were instructed to perform these tests before taking medication. Each measurement consisted of three manoeuvres and the readings were reported in a diary.

The diary was also used for reporting the occurrence of acute respiratory symptoms and medication use. Symptoms included in the diary were the following: cough, phlegm, runny/stuffed nose, woken up with breathing problems, shortness of breath, wheeze, attacks of shortness of breath with wheeze, fever, eye irritation and sore throat. Subjects had to indicate whether the symptom was absent, slight to moderate, or severe. Medication use was assessed by reporting a maximum of three names of the medication and the number of units taken.

Daily measurements of 24-hour average concentrations of particulate matter with an aerodynamic diameter less than 10 μm (PM10) and black smoke were measured in both winters in urban and control area from 3pm to 3pm. Detailed methods are published elsewhere [85]. In Amsterdam (urban area), $SO_2$ and $NO_2$ concentrations were obtained in both winters from continuous monitors of the municipal research agency for the environment and soil mechanics (OMEGAM). For the control areas, the concentrations of $NO_2$ and $SO_2$ were obtained from sites close by the study areas (that is, Barsbeek in the first winter and Witteveen in the second winter) both being part of the National Air Quality Monitoring Network.

The intake of the antioxidant vitamins C, E, and β-carotene was measured with a semi-quantitative food frequency questionnaire. This questionnaire was developed and validated to quantify, in particular, energy and antioxidant intake [86,87]. The structure of this food frequency questionnaire did not allow calculation of the nutrient contribution of vitamin supplements, such as vitamins C, E, A and multivitamins.

Blood specimens were stored in the refrigerator (4 °C) until centrifugation at the end of the day to obtain serum samples. Aliquots of serum were stored at –80 °C until analysis. From each subject, concentrations of α-tocopherol and β-carotene were measured simultaneously by reverse-phase high performance liquid chromatography (HPLC) in one run at Wageningen Agricultural University in the Netherlands. The method was modified as described by Hess and co-workers [75]. Duplicate samples (n=52) were measured to calculate the coefficient of variation of the HPLC-measurement for β-carotene (10.9%) and for α-tocopherol (3.0%).

***Data analysis***

Subjects were included in the analysis if they had performed PEF measurements and filled out the diary for respiratory symptoms in at least 60% of the total study period. In addition, the first two days of each subject were excluded from analysis because of a possible learning effect on the PEF measurements.

Before the analysis, the antioxidant variables were divided in high and low levels with a cut-off point at the median of intake or serum concentration of the antioxidants. Dietary intake of antioxidants was standardised to energy intake before calculating the median. To reduce misclassification of the dietary antioxidant intake, subjects who reported daily use of supplements of vitamins C, and A were classified in the highest category, that is above the median of dietary antioxidant intake. Subjects who used multivitamins were classified in the highest category of the dietary antioxidants.

Each of the four panels was divided into subgroups of high and low antioxidant levels. Within these subgroups, the prevalences of respiratory symptoms, bronchodilator use and PEF decrements of more than 10% or more than 20% below the subject-specific median for the morning and evening were calculated; the method for calculation of these prevalences of large PEF decrements has been published in detail elsewhere [88]. Next, the relations between these prevalences and the different air pollutants were calculated with logistic regression analysis [89,90]. The prevalences of the PEF decrements, respiratory symptoms or bronchodilater were used as dependent variables. The following respiratory symptoms were considered: cough, phlegm, lower respiratory symptoms (LRS) and upper respiratory symptoms (URS). LRS were defined as the presence of the following symptoms on a day: wheeze, shortness of breath, and/or attacks of shortness of breath with wheeze; URS: stuffed/runny nose and/or sore throat. Air pollutant concentrations (PM10, black smoke, $NO_2$, $SO_2$) of the same day (lag 0), the previous day (lag 1), the day before yesterday (lag 2), and a five-day moving average (mean 5) were used separately as independent variables in the logistic regression models. In each model the following adjustments were applied with panel-specific parameter estimates: the minimum temperature, day of the week (weekend or holiday versus weekday), a time trend with the day of the study (number of the day since the start of the study) and a squared and cubic term for the day of the study, mean influenza incidence of the last six days and the mean influenza incidence between day seven to day 13 prior to the reporting date. Data on influenza morbidity were obtained from the Netherlands Institute of Primary Health Care (NIVEL). This institute is running a registration network of 46 sentinel general practices (GP) covering about 1% of the Dutch population. Since the prevalence of PEF decrements, symptoms and medication use was correlated with the prevalence of the previous day, an adjustment for auto-

correlation was used in the regression models by specifying a first order autoregressive model for the residuals [89].

Finally, meta analysis techniques were used to obtain a combined effect estimate for a high versus a low antioxidant intake or serum level from the panel specific effect estimates. A statistical test of the homogeneity of the panel specific effect estimates was conducted using a chi-square test. Homogeneity was assumed if the p-value was greater or equal than 0.25 (conservative cut-off point) and the combined effect estimate was then calculated as a weighted average using the inverse of the variance of the panel specific regression slopes as the weights. In case of heterogeneity ($p<0.25$) the combined effect estimates were calculated using random effect estimation with the non-iterative method with unequal weights [91]. The results are presented as Odds Ratios (ORs) with 95% confidence intervals for an increase in PM10 of 100 $\mu g/m^3$ and for an increase in black smoke, $SO_2$ and $NO_2$ of 40 $\mu g/m^3$.

## Results

In the winter of 1993/1994, 168 subjects with chronic respiratory symptoms started the study. Of these, 46 were excluded from the analysis because 35 subjects did not have at least 60% of the total PEF measurements, 9 subjects did not provide blood samples and 2 subjects did not complete the food frequency questionnaire. In the winter of 1994/1995, 128 subjects started the study. Of these, 23 were excluded from the analysis because 16 subjects did not have at least 60% of the total PEF measurements and 7 subjects did not provide blood samples. So, a total of 227 subjects were available for analysis.

Table 1 describes relevant population characteristics and the mean intake and serum levels of antioxidants. The mean age of the study population was 60 years with an equal number of women and men. About 25% of the subjects used daily vitamin supplements. The subjects who were excluded from analysis (n=69) did not differ in gender, smoking status, total IgE levels or the use of vitamin supplements but they were slightly older (62 years) compared to subjects who were included in the analysis.

Preliminary data analysis showed that age, gender, smoking and IgE levels did not confound the relation between antioxidants (serum and dietary) and acute effects of air pollution on PEF. In addition, preliminary individual linear regression analysis did not show a modulation of the acute effects of winter air pollution on PEF by serum $\alpha$-tocopherol or dietary vitamin E. Therefore, the further presentation will focus on dietary vitamin C and $\beta$-carotene and serum $\beta$-carotene. After adjustment for energy intake of the dietary antioxidants, the Pearson's correlation coefficients between

dietary vitamin C and dietary β-carotene was 0.63 and between dietary vitamin C and serum β-carotene 0.18; the correlation coefficient between β-carotene in diet and serum was 0.31.

The air pollution concentrations in urban and non-urban areas were described in detail in another paper [85]. Briefly, the mean PM10 and black smoke

**Table 1:** *Mean (SD) characteristics and median intake and serum levels of antioxidants*

| Characteristics | Mean (SD) n=227 |
|---|---|
| Age (years) | 59.8 (6.3) |
| Gender (% women) | 50.9 |
| Smokers inside the house (%) | 27.3 |
| High total IgE (%)† | 18.5 |
| Vitamin supplement users (%)‡ | 25.1 |
| Dietary intake | Median (range) |
| Vitamin C (mg) | 119.8 (35 – 384) |
| Vitamin E (mg) | 13.8 (4.0 – 39.0) |
| β-carotene (mg) | 2.0 (0.5 – 6.1) |
| Serum concentrations | |
| α-tocopherol (μmol/l) | 36.0 (3.6 – 70.6) |
| β-carotene (μmol/l) | 0.30 (0.00 – 2.07) |

† High total IgE was defined as a level above 100 kU/l

‡ Percentage of subjects who daily used vitamin supplements of vitamins A, E and C and/or multivitamins

concentrations were slightly higher in the urban areas than in the non-urban areas and higher in the first winter compared to the second winter. Figure 1 shows the 24-hour mean concentrations for PM10 and black smoke in the urban area for the first winter; figure 2 shows the same for the second winter.

The overall effect of air pollution on respiratory symptoms and PEF decrements for the different panels will be described elsewhere. Briefly, PM10 and black smoke concentrations were positively associated with the prevalence of the PEF decrements larger than 20%, in particular, the morning PEF.

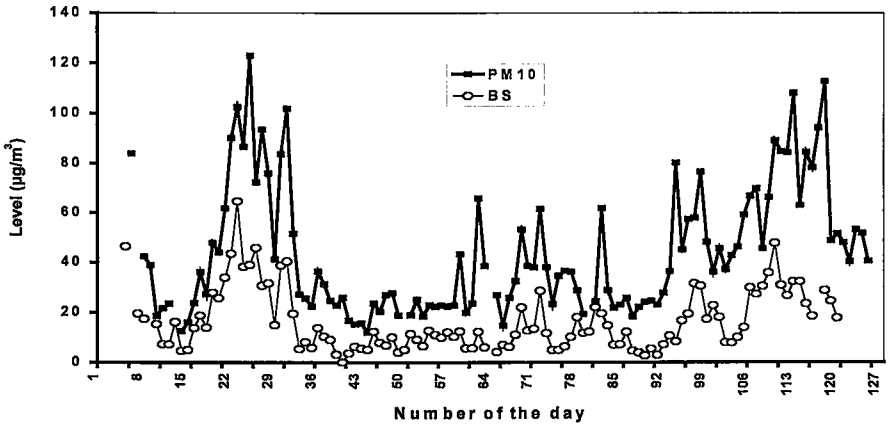


**Figure 1:** *Plot of the 24-hour mean PM10 and Black Smoke (BS) concentrations ($\mu g/m^3$) in the urban area for each day during winter 1993/1994*

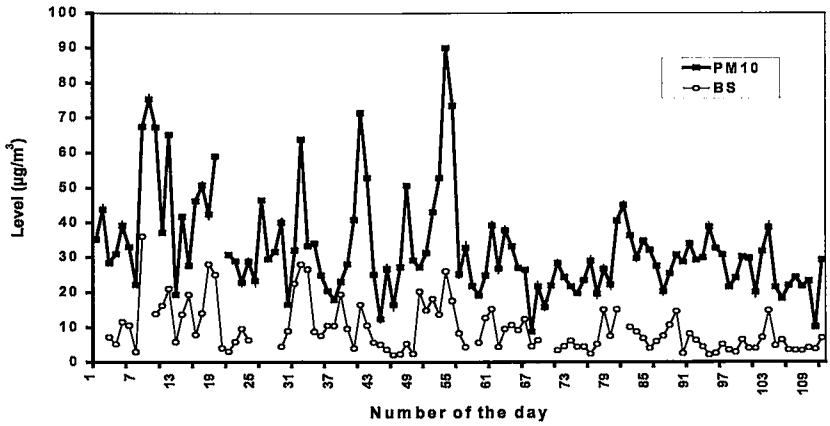


**Figure 2:** *Plot of the 24-hour mean PM10 and Black Smoke (BS) concentrations ($\mu g/m^3$) in the urban area for each day during winter 1994/1995*

**Table 2:** *Association [†] between the prevalence of PEF decrements of 10 and 20% and PM10 for high and low serum β-carotene concentration*

| | Low serum β-carotene (<0.30 μmol/l) | | | | High serum β-carotene (≥0.30 μmol/l) | | | |
|---|---|---|---|---|---|---|---|---|
| | PEF decrements | | | | PEF decrements | | | |
| | Morning | | Evening | | Morning | | Evening | |
| Range PEF | 10% | 20% | 10% | 20% | 10% | 20% | 10% | 20% |
| prevalences[‡] | 5.4-6.2 | 0.6-2.5 | 4.6-6.4 | 0.3-1.7 | 5.1-8.3 | 0.6-2.2 | 3.0-7.6 | 0.3-2.2 |
| Lag 0 | 1.41 | 3.26 | 1.14 | 2.56 | 0.91 | 0.91 | 1.09 | 0.20 |
| | (0.91-2.20) | (1.34-7.95) | (0.75-1.75) | (1.01-6.48) | (0.57-1.46) | (0.40-2.10) | (0.66-1.78) | (0.01-4.58) |
| Lag 1 | 1.62 | 3.43 | 1.06 | 1.81 | 0.98 | 0.54 | 0.91 | 2.93 |
| | (1.10-2.40) | (1.35-8.71) | (0.71-1.58) | (0.10-32) | (0.63-1.52) | (0.25-1.21) | (0.25-3.31) | (0.57-15) |
| Lag 2 | 1.16 | 0.86 | 0.95 | 6.25 | 0.80 | 0.63 | 0.62 | 0.55 |
| | (0.77-1.77) | (0.18-4.10) | (0.60-1.49) | (0.0-45602) | (0.53-1.19) | (0.14-2.78) | (0.24-1.59) | (0.19-1.66) |
| Mean 5 | 2.19 | 3.30 | 0.90 | 1.93 | 0.75 | 0.14 | 0.49 | 0.77 |
| | (1.14-4.19) | (0.40-27.2) | (0.50-1.61) | (0.27-13.9) | (0.36-1.57) | (0.03-0.67) | (0.06-4.04) | (0.03-23) |

[†] Presented as Odds Ratios (95% confidence intervals) for an increase in PM10 of 100 μg/m$^3$

[‡] Range in the mean PEF prevalences of each panel

Table 2 shows the associations between PM10 (increase of 100 μg/m$^3$) and the prevalence of PEF decrements larger than 10% or 20% in the morning and evening for subjects with high and low levels of serum β-carotene. The prevalences of PEF decrements of more than 20% were in most panels lower than 2%. Subjects with a low level of serum β-carotene had mostly odds ratios (ORs) above one, in particular, for the morning PEF decrements. Subjects with a high level of serum β-carotene, however, had mostly ORs which were not different from one. Table 3 shows that the effect of PM10 of the previous day on upper and lower respiratory symptoms or medication use was not different between subjects with high and low levels of serum β-carotene. The results were similar for different lags of PM10. In addition, the effect of PM10 on cough and phlegm was not different for a high versus a low serum β-carotene concentration.

**Table 3:** *Association [†] between the prevalence of respiratory symptoms or medication use and air pollution of the previous day for a high and a low serum β-carotene concentration*

| Air pollutant | Low serum β-carotene (<0.30 μmol/l) | | | High serum β-carotene (≥0.30 μmol/l) | | |
|---|---|---|---|---|---|---|
| | LRS | URS | medication use | LRS | URS | medication use |
| | Prevalences[‡] | | | Prevalences[‡] | | |
| | 16.2-34.8 | 25.5-36.8 | 4.5-18.4 | 15.3-20.2 | 19.3-42.0 | 7.1-16.6 |
| PM10 | 1.04 | 1.10 | 1.01 | 1.07 | 1.13 | 1.03 |
| | (0.92-1.18) | (0.95-1.27) | (0.86-1.18) | (0.91-1.26) | (0.97-1.31) | (0.92-1.16) |
| Black smoke | 1.10 | 1.17 | 0.98 | 0.93 | 1.16 | 1.06 |
| | (0.97-1.25) | (1.02-1.34) | (0.78-1.24) | (0.71-1.22) | (1.01-1.33) | (0.94-1.19) |

[†] Presented as an Odds Ratios (95% confidence intervals) for an increase in PM10 of 100 μg/m$^3$ and in black smoke of 40 μg/m$^3$

[‡] Range in mean prevalences of each panel for upper respiratory symptoms (URS), lower respiratory symptoms (LRS) and medication use

Table 4 shows the associations between black smoke (increase of 40 μg/m$^3$) and the prevalence of PEF decrements larger than 10% or 20% in the morning and evening for the groups with a high and low level of serum β-carotene. Subjects with a low level of serum β-carotene had mostly ORs above one, in particular, for a black smoke concentration of the same day, the previous day or a 5-day mean. Subjects with a high level of serum β-carotene had mostly ORs that did not differ from zero.

**Table 4:** *Association [†] between the prevalence of PEF decrements of 10 and 20% and black smoke for high and low serum β-carotene concentration*

| | Low serum β-carotene (<0.30 μmol/l) | | | | High serum β-carotene (≥0.30 μmol/l) | | | |
|---|---|---|---|---|---|---|---|---|
| | PEF decrements | | | | PEF decrements | | | |
| | Morning | | Evening | | Morning | | Evening | |
| Range PEF | 10% | 20% | 10% | 20% | 10% | 20% | 10% | 20% |
| prevalences[‡] | 5.4-6.2 | 0.6-2.5 | 4.6-6.4 | 0.3-1.7 | 5.1-8.3 | 0.6-2.2 | 3.0-7.6 | 0.3-2.2 |
| Lag 0 | 1.80 | 5.69 | 1.04 | 3.92 | 1.09 | 1.01 | 0.90 | 0.76 |
| | (1.14-2.82) | (2.49-13.0) | (0.70-1.56) | (1.67-9.20) | (0.72-1.66) | (0.52-1.97) | (0.48-1.72) | (0.28-2.04) |
| Lag 1 | 1.67 | 2.72 | 1.55 | 1.63 | 1.00 | 0.37 | 0.83 | 0.0 |
| | (1.15-2.43) | (1.36-5.45) | (0.71-3.38) | (0.0-1084) | (0.69-1.43) | (0.09-1.47) | (0.48-1.45) | $(0.0\text{-}5*10^{6})$ |
| Lag 2 | 1.14 | 0.68 | 0.86 | 0.18 | 0.88 | 0.22 | 0.86 | 0.09 |
| | (0.78-1.65) | (0.03-17.3) | (0.63-1.19) | (0.07-0.47) | (0.62-1.23) | (0.02-3.04) | (0.36-2.08) | (0.00-14.1) |
| Mean 5 | 3.90 | 31.5 | 1.05 | 35.1 | 0.76 | 0.76 | 0.30 | 0.12 |
| | (2.07-7.36) | (0.23-4214) | (0.62-1.76) | (5.96-206) | (0.32-1.81) | (0.25-2.35) | (0.04-2.25) | (0.02-0.79) |

[†] Presented as Odds Ratios (95% confidence intervals) for an increase in black smoke of 40 $\mu g/m^3$

[‡] Range in the mean PEF prevalences of each panel

Table 3 shows that the effect of black smoke of the previous day on upper and lower respiratory symptoms or medication use was not different between subjects with high and low levels of serum β-carotene. The results were similar for different lags of black smoke. In addition, the effect of black smoke on cough and phlegm was not different for a high versus a low serum β-carotene concentration.

The effect of $NO_2$ and $SO_2$ on large PEF decrements was slightly higher in the group with low serum β-carotene levels compared to the group of subjects with high serum β-carotene levels (results not shown).

The associations among PM10 of the previous day, 10% PEF decrements in the morning, lower respiratory symptoms and upper respiratory symptoms were not different for subjects with a low versus a high intake of vitamin C or β-carotene (table 5). The results were similar for different lags of PM10 en for black smoke, $NO_2$ and $SO_2$.

**Table 5:** *Association [†] between the prevalence of 10% morning PEF decrement, lower respiratory symptoms (LRS), upper respiratory symptoms (URS) and PM10 levels of the previous day (lag 1) for high and low intake of dietary antioxidants*

| | Low intake of antioxidants | | | High intake of antioxidants | | |
|---|---|---|---|---|---|---|
| Dietary | 10% PEF | LRS | URS | 10% PEF | LRS | URS |
| Vitamin C | 1.11 | 1.02 | 1.18 | 0.91 | 1.12 | 1.13 |
| | (0.79-1.56) | (0.88-1.19) | (1.00-1.39) | (0.60-1.37) | (0.97-1.30) | (0.99-1.30) |
| β-Carotene | 1.49 | 1.04 | 1.08 | 0.76 | 1.09 | 1.20 |
| | (0.87-2.55) | (0.90-1.21) | (0.88-1.32) | (0.44-1.29) | (0.95-1.25) | (0.99-1.46) |

[†] Presented as Odds Ratios (95% confidence intervals) for an increase in PM10 of 100 $\mu g/m^3$

## Discussion

This study shows that subjects with chronic respiratory symptoms and with low serum β-carotene levels were more likely to be affected by air pollution than those with high serum β-carotene levels were. This effect was most pronounced for the effect of particulate matter (PM10 or black smoke) on the prevalence of large PEF decrements. There was no difference in air pollution effects between high and low intake of vitamin C and β-carotene.

The results showed that the 95% confidence intervals were very wide for the ORs of air pollution on PEF decrements of more than 20%. These wide confidence intervals occurred because the prevalences of PEF decrements of more than 20% was small in each of the antioxidant groups within panels, often with a mean of less than 1% and a median of 0%.

Misclassification of the dietary antioxidants is a possible explanation for not finding a modifying effect of dietary antioxidants on the acute respiratory effects of air pollution. First because of measurement errors occur when the antioxidant vitamins

were calculated from the food frequency questionnaires [92]. Second because about 25% of the subjects used daily vitamin supplements. The nutrient contribution of these supplements could not be added to the intake of dietary antioxidants due to the structure of the food frequency questionnaire. To reduce the misclassification, we classified subjects with a daily use of vitamin supplements in the group of subjects with an intake above the median. So possibly due to the remaining misclassification, a modifying effect of dietary antioxidants would be biased towards the null.

Whether acute effects of air pollution are modulated by antioxidants can only be detected if the range in exposure to antioxidants is sufficient large in the population under study. In our study population, the range in dietary antioxidants was less then two between the 25$^{th}$ to 75$^{th}$ percentile. Dietary vitamin C had a range from 88 mg to 169 mg and dietary β-carotene had a range of 1.52 to 2.69 mg. These dietary ranges were relatively small compared to the range in serum β-carotene which was about 2.5 between the 25$^{th}$ (0.20 μmol/l) and 75$^{th}$ percentile (0.48 μmol/l) of serum β-carotene So, a more clear effect of dietary antioxidants might have occurred if the range in dietary antioxidants was larger in this study population.

Selection bias in the relation between air pollution and PEF is not very likely because in a panel study each subject is his/her own control in the exposure of air pollution. However, some selection could have occurred, since, more health conscious subjects were participating in the present study. This was observed because of the relatively high percentage of daily supplement users (25%). In a random sample of Dutch adults aged 20-59 years measured in 1994 and 1995, the percentage of daily supplement use (measured with the same questionnaire) was less than 10% [93]. Although our study population was slightly older (50-70 years), the number of supplement users in these older subjects was not different in the Dutch national Food consumption Survey in 1987-1988 compared to other adult age groups [94]. If subjects would take supplements because of experienced adverse health effects of air pollution, a bias towards a positive rather than a negative relation between antioxidants and acute respiratory effect of air pollution could occur. Since we did not find an effect of dietary antioxidants, the use of supplements was probably not associated with the experienced acute effects of air pollution.

Serum levels of β-carotene could be a better marker for antioxidant exposure in the lung tissue than the intake of β-carotene. This was confirmed in a small (n=21) study in which β-carotene was measured in lung tissues, serum, and in diet with a food frequency questionnaire [46]. The questionnaire was designed to measure both dietary and supplemental β-carotene. The Pearson's correlation coefficient between serum concentration of β-carotene and lung tissue (r=0.72) was higher than the correlations between dietary intake of β-carotene and β-carotene concentration in the

lung tissue (r=0.54). So the finding that we did find a modifying effect of serum β-carotene on acute effects of air pollution but not for dietary β-carotene might be because serum levels of β-carotene are better markers of the β-carotene levels in the lung.

To our knowledge, there has only been one other study investigating a possible modulation of the acute effect of winter air pollution on PEF but this study focused on vitamin C supplements [11]. In this cross-over study, subjects were policemen (n=20) directing traffic for four consecutive days receiving a vitamin C supplement (2 g) or placebo on each morning before work. Peakflow was measured at the beginning and at the end of the working day. The results suggest that the PEF decreased during working day in control group but not in the vitamin group. It should be noted that the levels of air pollution were unknown and that the dose of supplemental vitamin C could not be compared to the levels of dietary intake because it was 14 times the mean intake and 4 times the maximum intake of vitamin C in our study. So our study was the first study investigating dietary intake and serum levels of antioxidants in relation to the acute effects of winter air pollution.

Most other intervention studies under ambient conditions were investigating a possible modulation of the acute effect of ozone by antioxidant supplements. Two intervention studies were performed among cyclists performing lung function measurements before and after exercise in the Netherlands. In the first study in the summer of 1994, twelve subjects were supplemented with a daily cocktail of vitamin C (650 mg), vitamin E (75 mg), and β-carotene (15 mg) for 10 weeks. The control group (n=14) did not receive a placebo. An acute effect of ozone (8-hour mean 101 $\mu g/m^3$) was observed in the control group but not in the vitamin group [80]. The second study in the summer of 1996 was placebo-controlled. Half of the 38 cyclists received daily 100 mg vitamin E and 500 mg vitamin C for 15 weeks. Lung function ($FEV_1$ and FVC) was negatively associated with ozone concentration (8-hour mean 77 $\mu g/m^3$) in the placebo group but not in the vitamin group [83]. Street workers (n=49) in Mexico-city received the same cocktail as the cyclists of 1994 in the Netherlands in a placebo-controlled crossover study [31]. Although the street workers were not exercising heavily, the mean one-hour maximum ozone concentration was higher (110 ppb, equivalent to 163 $\mu g/m^3$ for conditions in Mexico City). The results of this study suggest that in particular in the first phase of the study the placebo group had a significant effect of ozone on $FEV_1$, FVC and MMEF while the supplementation group did not show an ozone effect. Since all these studies under ambient conditions supplemented their subjects (about 4 times the mean intake of vitamin C and about 7 times the mean intake of vitamin E and β-carotene), the question remains if the possible beneficial effect of antioxidants could be reached by a higher dietary intake alone.

The postulated mechanism for lung function decrements after ozone exposure is a reduction of maximal inspiratory capacity through stimulation of the neural receptors in the upper airways. Cyclo-oxygenase products of arachidonic acid stimulate these receptors which are released on exposure to ozone [67,95]. Vitamin C and E have been shown to affect this arachidonic acid metabolism but the role of antioxidants in this mechanism is not fully understood [20,68]. Another hypothesised mechanism of the acute effects of air pollution would be an increase in inflammatory mediators which was shown in the bronchoalveolar lavage within one hour [73,74], six hours [66], 18 hours [73,74], and 24 hours [66] after ozone exposure. Antioxidants could modulate the airway response to air pollution by reducing the influx of inflammatory cells [56]. Which of the two postulated mechanisms is relevant to the observed modulation in our study remains unclear because we did not measure inflammatory markers. To our knowledge, the mechanistic action of a possible protective role of β-carotene in relation to acute respiratory effects of air pollution has not been investigated.

In conclusion, the results of the present study suggest that serum β-carotene attenuated the respiratory effects of air pollution, in particular, the prevalence of large PEF decrements in relation to PM10 and black smoke in a panel of subjects with chronic respiratory symptoms. A higher intake of dietary vitamin C and β-carotene was not related to the acute effects of winter air pollution.

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# CHAPTER 5

# Dietary intake of antioxidant (pro-)vitamins, respiratory symptoms and pulmonary function: the MORGEN study


## Abstract

*Background* A study was undertaken to investigate the relations between the intake of the antioxidants (pro)-vitamins C, E and β-carotene and the presence of respiratory symptoms and lung function.

*Methods* Complete data were collected in a cross-sectional study in a random sample of the Dutch population on 6,555 adults during 1994 and 1995. Antioxidant intake was assessed by a semi-quantitative food frequency questionnaire. Respiratory symptoms (cough, phlegm, productive cough, wheeze, shortness of breath) were assessed by a self-administered questionnaire. Prevalence odds ratios for symptoms were calculated using logistic regression analysis. Linear regression analysis was used for forced expiratory volume in one second ($FEV_1$) and forced vital capacity (FVC). The results are presented as a comparison between the 90th and the 10th percentiles of antioxidant intake.

*Results* Vitamin C intake was not associated with most symptoms but was inversely related with cough. Subjects with a high intake of vitamin C had a 53 ml (95%CI: 23-83) higher $FEV_1$ and 79 ml (95%CI: 42-116) higher FVC than those with a low vitamin C intake. Vitamin E intake showed no association with most symptoms and lung function, but had a positive association with productive cough. The intake of β-carotene was not associated with most symptoms but had a positive association with wheeze. However, subjects with a high intake of β-carotene had a 60 ml (95%CI: 31-89) higher $FEV_1$ and 75 ml (95%CI: 40-110) higher FVC than those with a low intake of β-carotene.

*Conclusions* The results of this study suggest that a high intake of vitamin C or β-carotene is protective for $FEV_1$ and FVC compared with a low intake, but not for respiratory s ymptoms.


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## Introduction

Diet is a relatively new area of interest in the field of asthma and chronic obstructive pulmonary disease (COPD). Antioxidant vitamins are considered to be potentially protective factors in the respiratory system because antioxidants in the lung can scavenge endogenous and/or environmental oxidant sources [96-98].

A protective effect of fruit consumption has been reported for lung function [36,37] and for chronic non-specific lung disease (CNSLD) [16]. In cross-sectional studies a higher intake of vitamin C [13,40] was associated with larger lung volumes; a higher plasma concentration of vitamin C was associated with larger lung volumes in adults [14] but not in children [37], and a lower prevalence of wheeze and chronic bronchitis [18], suggesting a protective effect of vitamin C on respiratory disease in adults. The prospective Zutphen-study did not show an association between the intake of vitamin C or β-carotene and the incidence of CNSLD [16]. The Nurses Health Study showed no association between dietary vitamin C and the incidence of asthma but dietary β-carotene and vitamin E were inversely related with adult onset asthma [17]. In summary, no consistent pattern arises from these studies on the relation between antioxidant (pro-) vitamin intake and respiratory disease. To our knowledge no results on the relation between dietary β-carotene and lung function have been published.

The MORGEN study (the monitoring project on risk factors and health in the Netherlands) provided the opportunity to investigate the relations between the intake of the antioxidants (vitamins C, E or β-carotene) and the prevalence of a number of respiratory symptoms and lung function simultaneously.

## Methods

### *Study population*

The MORGEN study is a cross sectional investigation of the prevalence of risk factors for chronic diseases using self-administered questionnaires and a physical examination in a randomly selected sample of the Dutch population aged 20 to 59 years in three towns in the Netherlands (Amsterdam, Doetinchem and Maastricht). The average response rate of the three towns was 50%. A total of 8,695 subjects were enrolled in 1994 and 1995 for questionnaires and physical examination. Of these, 683 subjects did not perform a lung function measurement for practical reasons that operated randomly, such as non-availability of lung function devices, time constraints, etc. Of the remaining 8,012 subjects with lung function measurements, we excluded pregnant women (n=47), supplement users (n=688) and those with missing values on one or more of the confounders (n=722). Thus, the population for data analysis consisted of 6,555 individuals.

***Data collection***

Invitations to participate in the study were sent to a random sample of the population by municipal health services. Those subjects who agreed to participate received two self-administered questionnaires (general and semi-quantitative food frequency) and underwent a physical examination. The general questionnaire provided information about demographic variables, life style factors (smoking, physical activity, alcohol consumption), environmental factors (presence of pets, dampness of the house, indoor $NO_2$ sources), chronic respiratory symptoms and the presence of other chronic diseases (diabetes, migraine, low back pain, neck and shoulder pain). The physical examination included measurements of height, weight, waist-hip circumference, blood pressure, and lung function. Blood (non-fasting) samples were taken for determination of glucose, total and HDL-cholesterol.

For the present analyses we defined chronic respiratory symptoms as positive answers to the following questions: 'Do you cough when getting up during winter time on most days for at least three months a year?' (cough), 'Do you bring up phlegm when getting up during winter time on most days for at least three months a year?' (phlegm), 'Have you had productive cough for a period of three weeks in the last 3 years?' (productive cough), 'Have you been troubled by wheezing, not due to a cold or the flu, in the last twelve months?' (wheeze) and 'Are you short of breath when walking with other people of your own age on level ground?' (shortness of breath). 'Are you being woken by attacks of shortness of breath?' (nocturnal attacks of shortness of breath ). These questions on respiratory symptoms were selected from the Dutch part of the European Community Respiratory Health Survey [84,99].

Lung function was measured with a heated pneumotachometer (Jaeger, Germany). Calibration took place twice a day. Measurements were performed by trained paramedics. Subjects were seated in an upright posture with a fixed mouthpiece which was adjusted for the height of each individual and, in addition, a nose clip was used. A maximum of eight manoeuvres was performed. Subjects who did not achieve at least three technically acceptable of which two reproducible manoeuvres of BTPS corrected forced expiratory volume in one second ($FEV_1$) or forced vital capacity (FVC) according to ERS 1993 criteria [77] were excluded from either the $FEV_1$ or FVC analyses, or both. Analyses were based on the maximum value of the reproducible manoeuvres of $FEV_1$ and FVC. Pregnant women were not considered in data analysis because the actual lung function could have been attenuated [100].

The food frequency questionnaire was developed for the MORGEN study which is part of the Dutch cohort of the EPIC study (European Prospective Investigation into Cancer and Nutrition) [101]. The purpose of the questionnaire was, in

particular, to quantify energy and antioxidant intake [86,87]. The habitual consumption of 178 food items during the last year was calculated from the questionnaire. Nutrient and energy intake were quantified for each individual using an extended version of the 1993 computerised Dutch food composition table [102]. In 1991 and 1992 the reproducibility and relative validity of the food groups and nutrients were assessed in a validation study [86,87]. The structure of the food frequency questionnaire did not allow a calculation of the nutrient contribution of vitamin supplements. About 9% of the total study population had used daily vitamin supplements in the last 12 months (vitamin A, C, E and multivitamins; β-carotene is not a common constituent in any of the supplements in the Netherlands). Since this number of subjects was too small to be considered in separate analyses they were excluded from the data analysis to reduce possible misclassification of nutrient intake.

***Definition of variables***

The subjects were grouped into three categories according to their educational level: low (intermediate secondary education or less), intermediate (intermediate vocational or higher secondary education) and high (higher vocational or university education). Current smokers were defined as those smoking one or more cigarette(s) a day. Pack-years of smoking were defined for current and former smokers, one pack-year being equal to smoking 20 cigarettes a day for one year.

***Data analysis***

The shape of the relation between each antioxidant and respiratory symptoms or lung function was investigated by classifying the antioxidants into quintiles of intake. The cut-off points for each quintile were based on the distribution of the intake of subjects without any chronic respiratory symptoms. Since no essential deviation from linearity was observed, the intake of the antioxidant vitamins C, E and β-carotene were entered as continuous independent variables in logistic and linear regression models.

The presence of each respiratory symptom (cough, phlegm, productive cough, wheeze or shortness of breath, nocturnal attacks of shortness of breath) was used as the dependent variable in logistic regression analyses [60]. The independent variable of interest was the intake of antioxidants as a continuous variable. Prevalence Odds Ratios (ORs) with 95% confidence intervals (95% CI) were estimated using logistic regression analysis; ORs were presented as a comparison of the antioxidant intake in the 90th and 10th percentiles.

Models for $FEV_1$ and FVC were fitted with multiple linear regression [60]. To select a basic model for $FEV_1$ and FVC, taking account of gender, height and age, we considered several models using different powers of height and age. The choice

of the "best" model was based on assessment of model simplicity, analysis of residuals and the percentage of variance in $FEV_1$ and FVC explained by the model. We chose the following basic adjusted model: $FEV_1$ and FVC divided by height squared as dependent variable with age, age squared and gender as independent variables. Regression coefficients (in ml) were calculated for a standard height of 1.70 meter and were expressed as the difference in $FEV_1$ and FVC between subjects in the 90th and 10th percentiles of antioxidant intake.

The following confounding factors were considered as independent variables in the model: smoking status, pack years of smoking, educational level, town, energy intake (to standardise the intake of the antioxidants), body mass index (weight in kg divided by height in metres squared), alcohol consumption, physical activity (yes/no), the other two antioxidant (pro-)vitamins, medical treatment for hay fever (yes/no), and environmental factors such as the presence of pets (never/not anymore/currently present), dampness of the house by questions on the presence of damp or mould spots on the walls of homes during the last two years, gas cooking (yes/no), and presence of an unvented (gas-fired) water heater (yes/no) (as predominant indoor $NO_2$ source in homes). In the final models the following variables were adjusted for: age, gender, energy intake, smoking status and pack years of smoking. Adjustment for educational level was considered to be an over-adjustment in the relation between antioxidants and lung function or respiratory symptoms so we did not adjust for educational level. We were not able to perform statistical evaluation of the presence of effect modification of smoking status on the relation between antioxidants and lung function or respiratory symptoms because of the small numbers in each group. In addition, we could not study the independent effect of the intake of vitamin C and β-carotene adjusting for each other because these two antioxidants are present in the same food groups, such as fruits and vegetables resulting in a relatively high Spearman correlation coefficient (r=0.60).

## Results

Of the 6,555 subjects available for analyses, 6,103 had at least three technically acceptable lung function manoeuvres of whom 5,740 subjects had reproducible measurements for $FEV_1$ and 5,633 subjects for FVC.

Table 1 shows the characteristics of the total study population for subjects with and without reproducible $FEV_1$ measurements. We note that the subjects without reproducible $FEV_1$ measurements consisted of those who could not perform three technically acceptable measurements (n=452) plus subjects who met acceptability criteria but not the reproducibility criteria (n=363). The characteristics for subjects with and without reproducible FVC measurements were similar to those

subjects with and without reproducible $FEV_1$ measurements, therefore we only present the latter.

**Table 1:** *Mean (SD) characteristics for total population (N=6,555) and for subjects with and without technically acceptable and reproducible measurements of $FEV_1$*

| Characteristics | Total population (N=6,555) | Reproducible $FEV_1$ (N=5,740) | Non-reproducible $FEV_1$ ‡ (N=815) |
|---|---|---|---|
| Age (yr.) | 42.1 (11.0) | 41.7 (10.9) | 44.7 (10.9) |
| Height (m) | 1.72 (0.094) | 1.72 (0.093) | 1.69 (0.097) |
| Gender (% women) | 52.3 | 51.9 | 55.6 |
| Smoking status (%) | | | |
| Current smokers | 32.4 | 32.7 | 30.6 |
| Former smokers | 31.2 | 31.6 | 28.5 |
| Never smokers | 36.3 | 35.7 | 41.0 |
| Pack-years * | 17.4 (15.4) | 17.2 (15.1) | 18.8 (16.9) |
| Educational level (%) | | | |
| Low | 48.2 | 46.2 | 62.7 |
| Intermediate | 29.3 | 30.3 | 21.6 |
| High | 22.5 | 23.5 | 15.7 |
| BMI ($kg/m^2$) | 25.4 (3.9) | 25.3 (3.9) | 26.2 (4.3) |
| Physical Activity (%) | 64.5 | 65.8 | 55.4 |
| Alcohol use (%yes) | 60.9 | 62.2 | 51.4 |
| Respiratory symptoms † (%) | 32.7 | 32.1 | 36.5 |

* excluding those subjects who never smoked

† one or more of the following respiratory symptoms: cough, phlegm, productive cough, wheeze, shortness of breath, nocturnal attacks of shortness of breath, ever asthma

‡ including those subjects who were not able to perform at least three lung function manoeuvres (n=452)

For the total population, the mean age was 42 years; approximately one third of the study population were current smokers and about half had a low educational level. Subjects without reproducible $FEV_1$ measurements were older, had a lower educational level and were less physically active but included more never smokers and less alcohol consumers. Table 2 shows that the mean energy and nutrient intake was not different in subjects with or without reproducible $FEV_1$ measurements; the same was observed for non-reproducible versus reproducible FVC measurements (results not shown).

**Table 2:** *Mean (SD) energy and nutrient intake per day of nutrients for the total population (N=6,555) and for subjects with and without technically acceptable and reproducible measurements of $FEV_1$*

| | Total | Reproducible $FEV_1$ | Non-reproducible $FEV_1$ † |
|---|---|---|---|
| | (n=6,555) | (n=5,740) | (n=815) |
| Nutrients | Mean (SD) | Mean (SD) | Mean (SD) |
| Energy (MJ) | 9.8 (2.9) | 9.8 (2.9) | 9.7 (3.1) |
| Protein (en%) * | 15.3 (2.3) | 15.3 (2.3) | 15.3 (2.6) |
| Fat (en%) * | 35.7 (5.2) | 35.7 (5.2) | 35.6 (5.4) |
| Carbohydrates (en%) * | 45.2 (6.4) | 45.1 (6.4) | 45.6 (6.7) |
| Alcohol (en%) * | 3.6 (4.6) | 3.6 (4.6) | 3.2 (4.9) |
| Vitamin C (mg) | 132.6 (61.7) | 132.6 (61.7) | 132.5 (62.1) |
| Vitamin E (mg) | 16.3 (6.0) | 16.3 (5.9) | 16.1 (6.2) |
| β-Carotene (mg) | 2.33 (1.11) | 2.34 (1.10) | 2.28 (1.15) |

* protein, fat, carbohydrates and alcohol are expressed as a percentage of energy intake

† including those subjects who were not able to perform at least three lung function manoeuvres (n=452)

Possible confounding factors, such as gender, smoking status and educational level were evaluated. The intake of antioxidants was found to be related to these factors. Women had a higher intake of vitamin C and β-carotene but a lower intake of vitamin E than men, and current smokers had a lower intake of vitamin C and β-carotene but a higher intake of vitamin E than never smokers. The mean intake of the antioxidants was highest in the highest educational level. The dependent variables (respiratory symptoms, $FEV_1$ and FVC) were also related to these factors. The prevalence of the respiratory symptoms was higher and the mean $FEV_1$ and FVC was lower in current smokers than never smokers. Lung function was also associated with educational level, with those in the high education category having a better lung function. However, the prevalence of respiratory symptoms was not consistently different between educational levels.

The unadjusted prevalence of respiratory symptoms for each quintile of antioxidant intake and the unadjusted and adjusted mean of $FEV_1$ and FVC for each quintile of antioxidant intake is presented in table 3. There is no deviation from linearity.

**Table 3:** *Prevalence of respiratory symptoms (%) and mean FVC (L) and $FEV_1$ (L) by quintiles of antioxidants*

| | Quintiles† of vitamin C intake | | | | | Quintiles† of vitamin E intake | | | | | Quintiles† of β-carotene intake | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| Number of subjects | 1392 | 1314 | 1249 | 1263 | 1335 | 1247 | 1333 | 1249 | 1330 | 1396 | 1319 | 1283 | 1295 | 1262 | 1396 |
| Cough (%) | 10.2 | 7.0 | 6.4 | 4.7 | 5.3 | 6.3 | 7.1 | 6.4 | 6.0 | 8.0 | 7.8 | 6.9 | 7.1 | 5.6 | 6.3 |
| Phlegm (%) | 7.9 | 6.1 | 6.8 | 5.0 | 4.8 | 5.0 | 6.5 | 5.7 | 6.3 | 7.0 | 6.8 | 6.9 | 5.4 | 4.8 | 6.8 |
| Prod. Cough (%) | 17.3 | 17.6 | 16.0 | 16.9 | 19.1 | 14.1 | 16.3 | 17.2 | 18.6 | 20.5 | 16.6 | 15.2 | 17.5 | 17.0 | 20.4 |
| Wheeze (%) | 10.8 | 7.7 | 7.8 | 6.7 | 8.8 | 9.0 | 8.9 | 7.7 | 7.1 | 9.2 | 8.0 | 9.3 | 7.1 | 7.6 | 9.9 |
| SOB (%) | 9.0 | 4.4 | 5.7 | 3.4 | 5.9 | 7.6 | 5.8 | 4.5 | 5.2 | 5.5 | 6.7 | 6.3 | 4.9 | 5.5 | 5.2 |
| Noct. Attacks SOB (%) | 6.0 | 5.5 | 4.7 | 4.9 | 5.6 | 5.8 | 6.5 | 4.3 | 4.2 | 6.0 | 5.1 | 5.9 | 4.7 | 4.7 | 6.5 |
| Number of subjects | 1199 | 1170 | 1103 | 1114 | 1154 | 1069 | 1164 | 1117 | 1168 | 1222 | 1131 | 1118 | 1141 | 1111 | 1239 |
| $FEV_1$ (L), unadjusted | 3.50 | 3.63 | 3.58 | 3.65 | 3.62 | 3.29 | 3.48 | 3.60 | 3.72 | 3.84 | 3.44 | 3.53 | 3.62 | 6.50 | 3.72 |
| $FEV_1$ (L), adjusted * | 3.38 | 3.50 | 3.47 | 3.52 | 3.52 | 3.43 | 3.48 | 3.48 | 3.50 | 3.48 | 3.41 | 3.44 | 3.49 | 3.51 | 3.53 |
| Number of subjects | 1191 | 1150 | 1072 | 1080 | 1140 | 1051 | 1143 | 1100 | 1141 | 1198 | 1116 | 1105 | 1124 | 1080 | 1208 |
| FVC (L), unadjusted | 4.48 | 4.60 | 4.56 | 4.64 | 4.56 | 4.20 | 4.40 | 4.55 | 4.76 | 4.89 | 4.41 | 4.50 | 4.62 | 4.64 | 4.68 |
| FVC (L), adjusted * | 4.29 | 4.42 | 4.41 | 4.47 | 4.47 | 4.34 | 4.41 | 4.40 | 4.45 | 4.44 | 4.33 | 4.36 | 4.44 | 4.45 | 4.46 |

SOB = shortness of breath

* mean $FEV_1$ and FVC adjusted for age, age squared and gender, presented for a height of 1.70 m

† the cut-off points for each quintile were based on the distribution of the intake of subjects without any chronic respiratory symptoms

**Table 4:** *The relation between antioxidants (vitamin C, vitamin E, and β-carotene) and respiratory symptoms or lung function (N=6,555)*

| Dependent variable | Vitamin C | | | Vitamin E | | | β-carotene | | |
|---|---|---|---|---|---|---|---|---|---|
| | OR * | OR † | 95% CI † | OR * | OR † | 95% CI † | OR * | OR † | 95% CI † |
| Respiratory symptoms | | | | | | | | | |
| Cough (n=6533) | 0.53 | 0.66 | 0.50 to 0.87 | 1.28 | 0.85 | 0.61 to 1.18 | 0.79 | 0.86 | 0.67 to 1.10 |
| Phlegm (n=6541) | 0.67 | 0.77 | 0.59 to 1.02 | 1.32 | 1.06 | 0.76 to 1.47 | 1.04 | 1.11 | 0.87 to 1.40 |
| Prod. Cough (n=6536) | 1.08 | 1.09 | 0.93 to 1.28 | 1.47 | 1.26 | 1.02 to 1.56 | 1.20 | 1.14 | 0.99 to 1.33 |
| Wheeze (n=6514) | 0.87 | 1.04 | 0.83 to 1.30 | 1.18 | 1.13 | 0.85 to 1.52 | 1.15 | 1.27 | 1.04 to 1.55 |
| SOB (n=6494) | 0.75 | 0.81 | 0.61 to 1.07 | 0.88 | 1.24 | 0.87 to 1.77 | 0.87 | 1.00 | 0.77 to 1.29 |
| Noct. Attacks SOB (n=6539) | 0.90 | 0.95 | 0.72 to 1.25 | 1.10 | 1.20 | 0.84 to 1.71 | 1.16 | 1.22 | 0.96 to 1.56 |
| Lung function | Diff ‡ | Diff § | 95% CI § | Diff ‡ | Diff § | 95% CI § | Diff ‡ | Diff § | 95% CI § |
| $FEV_1$ (n=5740) | 91.1 | 52.9 | 23.0 to 82.3 | 33.0 | 27.9 | -12.9 to 68.7 | 83.2 | 60.0 | 31.4 to 88.6 |
| FVC (n=5633) | 117.8 | 79.0 | 42.3 to115.7 | 66.3 | 18.2 | -32.2 to 68.6 | 105.4 | 75.2 | 40.2 to110.2 |

SOB = shortness of breath

* unadjusted prevalence odds ratios (OR), presented for subjects in the 90$^{th}$ percentile versus those subjects in the 10$^{th}$ percentile of antioxidant intake – that is, for vitamin C intake 144.9 mg, for vitamin E intake 14.4 mg and for β-carotene intake 2.50 mg

† prevalence odds ratios (with 95% Confidence Interval) adjusted for age, gender, energy intake, smoking status, pack-years of smoking, presented for subjects in the 90$^{th}$ percentile versus those subjects in the 10$^{th}$ percentile of antioxidant intake

‡ difference in $FEV_1$ and FVC (in ml for a standard height of 1.70 m) between subjects in the 90$^{th}$ percentile and those in the 10$^{th}$ percentile of antioxidant intake adjusted for age, age squared, gender

§ difference in $FEV_1$ and FVC (in ml for a standard height of 1.70 m) with 95% Confidence Interval (95% CI) between subjects in the 90$^{th}$ percentile and those in the 10$^{th}$ percentile of antioxidant intake adjusted for age, age squared, gender, energy intake, smoking status, pack-years of smoking

The results of logistic regression analysis with the intake of antioxidants as a continuous variable are presented in table 4. The unadjusted and adjusted ORs for respiratory symptoms and the difference for $FEV_1$ and FVC represent the comparison of subjects in the 90$^{th}$ percentile with those in the 10$^{th}$ percentile of antioxidant intake. After adjustment for the considered confounding factors, the ORs of vitamin C intake with most of the symptoms were around 1. The OR of vitamin C with cough was statistically significantly below 1 (OR=0.66; 95% CI: 0.50-0.87). $FEV_1$ was 53 ml (95% CI: 23 to 82 ml) higher in subjects with a high intake of vitamin C than in subjects with a low intake; for FVC the difference was 79 ml (95% CI: 42 to 116 ml). After adjustment, the ORs of vitamin E intake with symptoms were around 1, with a significantly increased OR for productive cough (OR=1.26; 95% CI: 1.02-1.56). Vitamin E intake was not associated with $FEV_1$ and FVC. The adjusted ORs of the intake of β-carotene with symptoms were mostly around 1 with the exception of a significantly increased OR for wheeze (OR=1.27; 95% CI: 1.04-1.55). However, $FEV_1$ was 60 ml (95% CI: 31 to 89 ml) higher in subjects with a high intake of β-carotene than in subjects with a low intake; for FVC the difference was 75 ml (95% CI: 40 to 110 ml).

The associations between the intake of vitamin C or β-carotene and lung function did not change after adjustment for the intake of vitamin E.

## Discussion

In the present study we observed that a high intake of vitamin C and β-carotene, but not vitamin E, was associated with a higher $FEV_1$ and FVC than a low intake of these antioxidants. No consistent associations were observed with respiratory symptoms. This suggests that dietary vitamin C and β-carotene have a protective effect on lung function but not on respiratory symptoms.

Lung function can be considered as a more objective measurement than respiratory symptoms. The lack of protective effect of vitamin C and β-carotene on respiratory symptoms might be due to reporting bias or due to an altered diet in those with respiratory symptoms. Another reason for the lack of agreement between the results on lung function and respiratory symptoms could be that the relevant lag time for a possible protective effect of antioxidants on lung function differs from that for respiratory symptoms.

Educational level was associated with the intake of antioxidants and with lung function but not with respiratory symptoms. This was not observed in other studies [13-15,18]. However, the present study showed that, after adjustment for educational level, the estimated effect between antioxidants and lung function decreased which suggest that educational level is a confounding factor. Since subjects in the high

educational level are more likely to have a healthy life style which correlates also with a higher intake of antioxidants, we considered that educational level would be a healthy life style indicator which would lead to over-adjustment of the relation between antioxidants and lung function. Other more specific healthy life style factors, such as physical activity, alcohol consumption and body mass index, did not materially affect the relation between antioxidants and lung function.

The associations between antioxidant intake and respiratory symptoms or lung function may have been biased towards the null due to misclassification of exposure. As with most dietary assessment methods, semi-quantitative food frequency questionnaires have a tendency to random misclassification. In the present study, we used a semi-quantitative food frequency questionnaire with correlations similar to those of other validated food frequency questionnaires [87,103-106]. However, the reproducibility and relative validity are often low leading to attenuation of the observed associations.

Subjects who did not meet ERS criteria for technically acceptable and reproducible lung function manoeuvres were excluded from the analyses on dietary antioxidants and lung function. This raises the question of selection bias. The relation between antioxidants and respiratory symptoms was, however, not materially different between the total group and in the total group excluding subjects who did not meet ERS criteria. Although selection bias can not be totally excluded in the relation between antioxidants and lung function, it does not seem very likely in this study.

The results of the present study with respect to the intake of antioxidants and respiratory symptoms can only be crudely compared with other studies because respiratory symptoms or disease as outcome were not completely comparable.

We did not find an association between most symptoms and the intake of vitamin C, only cough was significantly negatively associated with vitamin C. In the Nurses Health Study [17], dietary vitamin C was not associated with the incidence of asthma. NHANES II [18] did not show an association between dietary vitamin C and wheeze, but dietary vitamin C was associated with the presence of current bronchitis. A protective effect of serum vitamin C was observed with wheeze and current bronchitis [18].

We found that a high intake of vitamin C was associated with a 53 ml higher $FEV_1$ and 79 ml higher FVC than a low intake. This was consistent with the results of other studies investigating the intake of vitamin C [13,15,40] or plasma levels of vitamin C [14] with lung function. Schwartz and Weiss [40] and Britton and co-workers [13] showed that a higher intake of vitamin C was associated with a higher $FEV_1$; the size of the effect was of the same order of magnitude as in the present study [40]. The magnitude

of the association between vitamin C and FVC in the study of Britton and co-workers [13] was also comparable to that of the present study. Dow and co-workers [15] investigated the association between the intake of vitamin C with $FEV_1$ and FVC. After additional adjustment for vitamin E, the associations were of the same order of magnitude as in the present and in those of Britton *et al* [13] and Schwartz and Weiss [40], but were not statistically significant possibly because of the small sample size (N=178). In summary, these studies suggest a protective effect of vitamin C intake on lung function but not on symptoms or disease as outcome.

The intake of vitamin E was not associated with most of the symptoms or with lung function. This is consistent with the study of Britton and co-workers [13] which showed that the intake of vitamin E was not associated with $FEV_1$ or FVC independent of the intake of vitamin C. In contrast, Dow and co-workers [15] showed that the intake of vitamin E was positively associated with lung function independent of the intake of vitamin C. Troisi and co-workers observed that a higher intake of vitamin E was significantly associated with a lower incidence of asthma [17]. Thus, the results of these few studies are not consistent.

The intake of β-carotene was not associated with the prevalence of symptoms in the present study. This is consistent with the Nurses Health Study which showed no association between the intake of carotene and the incidence of asthma [17]. However, we observed that the intake of β-carotene was positively associated with $FEV_1$ and FVC. To our knowledge, no results on dietary β-carotene in relation with lung function have been published. The intake of total carotene was not associated with $FEV_1$, FVC and the ratio of $FEV_1$/FVC among 10,416 subjects in a cross-sectional study of Shahar and co-workers [42]. They pointed out, however, that the different carotenoids may have different effects on lung physiology. Blood levels of β-carotene were related to lung function in two studies. A high level of β-carotene was not associated with airway obstruction (N=83) [41]. However, the pilot phase of the CARET-study among 816 men exposed to asbestos showed that serum β-carotene was positively associated with $FEV_1$ and FVC [43].

In summary, the present study has shown that the intake of vitamin C and β-carotene has a protective effect on lung function but not on respiratory symptoms. The findings on the intake of vitamin C are consistent with those of other studies. The intake of vitamin E was not associated with respiratory symptoms or lung function which was not completely consistent with the findings of other studies.

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# CHAPTER 6

# Plasma antioxidant (pro-) vitamins in relation to respiratory symptoms in non-smokers: a case-control study

**Abstract**

*Background* The aim of this case-control study was to investigate the relations between plasma levels of antioxidants, β-carotene and α-tocopherol, and chronic respiratory symptoms in a group of Dutch adults (20-59 yrs) who were never or long-term former smokers.

*Methods* A case-control sample was selected from a population-based cross-sectional study and plasma concentrations of antioxidants were determined in 491 cases and 496 controls. Cases were subjects who reported one or more respiratory symptoms. Odds ratios (ORs) were calculated for the chronic respiratory symptoms using logistic regression analysis and are presented for a difference between the 90$^{th}$ percentile (high) and the 10$^{th}$ percentile (low) of plasma antioxidant concentration. ORs were adjusted for age, gender and body mass index.

*Results* The adjusted OR of plasma β-carotene for respiratory symptoms was below one (OR=0.81; 95%CI: 0.63-1.04). The OR of plasma α-tocopherol was slightly elevated for respiratory symptoms (OR=1.36; 95% CI 0.98-1.89) but this was mostly because of the elevated OR for dyspnea (OR=2.10; 95%CI: 1.28-3.43). Dyspnea is, however, also a common symptom in cardiovascular disease. Additional adjustment for cardiovascular risk factors did not change the positive association between dyspnea and plasma α-tocopherol.

*Conclusions* Cases tended to have lower plasma β-carotene levels than controls. Plasma α-tocopherol was not associated with asthma and chronic bronchitis symptoms but was positively associated with dyspnea.

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## Introduction

Antioxidant (pro-)vitamins, such as vitamins C, E and β-carotene, are considered to be beneficial for respiratory disease [97,98] because of a possible prevention of lung tissue damage by oxidant exposure and/or oxidative processes of inflammation [96].

So far most studies were on dietary antioxidants measured by questionnaire in relation to respiratory disease [16,17,19,93]. However, blood concentration of antioxidants is an additional marker of the nutritional status [48,96]. This marker is probably also closer related to antioxidant concentration in the lung tissue compared to dietary antioxidants measured by questionnaire [46,107]. To our knowledge, there has only been one study on blood levels of antioxidants in relation to respiratory symptoms [19]. In a cross-sectional analysis, serum levels of β-carotene and α-tocopherol were associated with a lower prevalence of COPD symptoms in smokers. However, an intervention study within the same publication did not find a change in the incidence of COPD symptoms after supplementation of α-tocopherol or β-carotene for six years in smokers [19].

We chose to study the relation between plasma antioxidants (α-tocopherol and β-carotene) and respiratory symptoms. At the time we designed our study, no other finding had been published on this relation, so we decided first to study this relation in a group of non-smokers. Cigarette smoking as a strong determinant of respiratory symptoms additionally affects the antioxidant status [108] and a relation between antioxidants and respiratory symptoms could, therefore, be more difficult to establish among smokers.

## Methods

### *Study population*

The MORGEN-study (the monitoring project on risk factors and health in the Netherlands) is a cross-sectional investigation on the prevalence of risk factors for chronic diseases in a randomly selected sample of the Dutch population aged 20 to 59 years in three towns in the Netherlands (Amsterdam, Doetinchem and Maastricht). We chose for a case-control design based on subjects with and without chronic respiratory symptoms. Subjects were eligible for this case-control sample if they were not pregnant and were non-smokers (defined as never and long-term former smokers who stopped smoking cigarettes more than 10 years ago) to avoid a possible recent exposure of high levels of oxidants. First, we determined that a sample size of 1,000 subjects (500 cases and 500 controls) would allow us to detect an Odds Ratio of two (fifth versus first quintile of antioxidants) at a 5% significance level with a statistical power of 80%. Subsequently, cases were selected from the most recent study period (before May 1995). Between July 1994 and May 1995, 521

eligible subjects who reported one or more chronic respiratory symptoms were defined as cases. A random sample of 527 controls was drawn from 1,266 eligible non-cases during the same period. Plasma concentrations of α-tocopherol and β-carotene were determined in these cases and controls.

***Data collection***

All respondents from the MORGEN-project filled out a general and a semi-quantitative food frequency questionnaire [86,87] and underwent a physical examination. The general questionnaire provided information about demographic variables, life-style factors, environmental factors, respiratory symptoms and the presence of chronic diseases. The questions on respiratory symptoms were selected from the Dutch part of the European Community Respiratory Health Survey (see appendix) [84,99]. The food frequency questionnaire assessed energy intake, nutrients and the self-reported use of diets for diabetes and for fat and cholesterol restriction. A physician or dietician did not necessarily prescribe these diets. The physical examination included measurements of height, weight, lung function, and collection of non-fasting blood (42 ml). Glucose and plasma total cholesterol was measured in all subjects routinely and the rest of the blood samples was stored. Plasma antioxidants (α-tocopherol and β-carotene) were determined in the selected case-control sample. The relation between plasma antioxidants (α-tocopherol and β-carotene) and lung function will be investigated in a random sample because the present case-control sample is not an appropriate study design to explore this cross-sectional relation.

Cases were defined as subjects who reported one or more respiratory symptoms. Controls were drawn from those subjects who did not report any chronic respiratory symptoms. In additional analysis, the respiratory symptoms were considered separately in three groups of cases: cases with asthma symptoms (wheeze with shortness of breath, wheeze without a cold, attacks of shortness of breath, ever asthma), cases with chronic bronchitis symptoms (chronic cough, chronic phlegm, productive cough) excluding cases with asthma symptoms, and cases with dyspnea as a symptom for emphysema (shortness of breath when walking actively, shortness of breath when walking compared to persons of the same age) excluding cases with asthma and chronic bronchitis symptoms.

Lung function measurements were used for an additional, more stringent definition of cases and controls. The measurements were performed with a heated pneumotachometer that was calibrated (Jaeger, Germany) [93]. Subjects were seated in an upright posture with a fixed mouthpiece, which was adjusted for the height of each individual. In addition, a noseclip was used. Subjects had to achieve at least

three technically acceptable, of which two reproducible, maneuvers of BTPS corrected $FEV_1$ according to ERS 1993 criteria [77].

Blood specimens were collected in ethylene diamine tetraacetic acid (EDTA) vacutainer tubes and stored in the refrigerator shielded from the light. Within four hours after collection, the blood was centrifuged to obtain plasma. Aliquots of plasma were stored initially at -20 °C. After six months or less, plasma was stored at -80 °C to secure the stability of the antioxidants [109]. Concentrations of α-tocopherol and β-carotene in each subject were measured by reverse-phase high-performance liquid chromatography (HPLC) in one run. The method was modified as described by Hess and co-workers [75]. About 7% (n=77) of the samples were measured in duplicate. The within-duplo coefficient of variation was 4.1% for plasma β-carotene and 3.1% for plasma α-tocopherol. Due to reasons that occurred randomly, such as not having enough plasma or a defective HPLC, plasma antioxidant levels could not be determined in 30 cases and 31 controls.

***Data analysis***

Presence or absence of one or more respiratory symptoms was used as the dependent variable in logistic regression analyses [60]. The independent variable of interest was the plasma concentration of β-carotene or α-tocopherol. First, these concentrations were analyzed in quintiles to study linearity with the lowest quintile as a reference. Cut-off points for each quintile were based on the distribution of the plasma antioxidants in the controls. Second, plasma antioxidant concentration was entered as a continuous independent variable in the model. The results of the latter analysis will be presented for a difference between the $90^{th}$ percentile (high) and the $10^{th}$ percentile (low) of the plasma antioxidant levels. Odds Ratios (ORs) with 95% Confidence Intervals (95% CI) were estimated.

Adjustment for plasma total cholesterol is common in studies with cardiovascular disease as an endpoint and plasma α-tocopherol as exposure variable because plasma total cholesterol is a confounding factor in this relation. We also considered adjustment for plasma total cholesterol in the present study. However, plasma total cholesterol is not a known risk factor for lung disease. So, we did not adjust for plasma total cholesterol in the relation between plasma α-tocopherol and respiratory symptoms.

Additional analysis with $FEV_1$ for a more stringent case-control definition was performed to increase the contrast between cases and controls. Of the 491 cases and 496 controls with plasma levels of antioxidants, 47 cases and 39 controls did not perform lung function measurements for practical reasons (non-availability of lung function devices, time constraints, etc.) that operated randomly and 69 cases and 59 controls did not fulfill ERS criteria [77]. So, 375 cases and 398 controls were available

for additional case-control definition with $FEV_1$. Cases were restricted to those with an $FEV_1$ equal or less than 90% of the predicted $FEV_1$ value and controls with an $FEV_1$ of equal or more than 100% resulting in 106 cases and 199 controls. Predicted $FEV_1$ values were calculated with multiple linear regression [60] with the following model: $FEV_1$ divided by height squared as dependent variable with age, age squared and gender as independent variables. The prediction equation was derived from all technically acceptable and reproducible lung function maneuvers of healthy never smokers without chronic respiratory symptoms (n=1,518) examined in 1994 and 1995 [93].

## Results

Table 1 shows that cases were older, included more women, had a higher body mass index (BMI), pack-years of smoking and a lower educational level than controls.

**Table 1:** *Mean characteristics and plasma antioxidant concentrations in cases and controls*

| Characteristics | Cases (n=491) | Controls (n=496) |
|---|---|---|
| | Mean (SD) | Mean (SD) |
| Age (yrs) | 43.3 (11.7) | 41.0 (11.8) * |
| Gender (% women) | 59.9 | 51.2 * |
| Smoking history (% former smokers) | 30.5 | 28.0 |
| Pack-years of past smoking † | 11.1 (11.9) | 8.1 (8.9) * |
| Educational level (%) ‡ | | |
| Low | 48.8 | 40.7 * |
| Intermediate | 27.3 | 32.3 |
| High | 24.0 | 27.0 |
| BMI ($kg/m^2$) | 26.5 (4.5) | 25.0 (3.7) * |
| Physical activity (% yes) | 65.8 | 68.7 |
| Alcohol use (% yes) § | 53.6 | 57.7 |
| Supplement use (% yes) ‖ | 7.1 | 6.7 |
| Plasma β-carotene (μmol/l) | 0.35 (0.21) | 0.37 (0.30) |
| Plasma α-tocopherol (μmol/l) | 31.2 (9.3) | 29.3 (7.6) * |

* $p < 0.05$ difference between cases and controls

† calculated only for long-term former smokers; one pack-year is equal to smoking 20 cigarettes a day for one year

‡ low (intermediate secondary education or less), intermediate (intermediate vocational or higher secondary education) and high (higher vocational or university education)

§ percentage of subjects whom drinks one or more glass per week

‖ daily vitamin supplement use of vitamin A, C, E and/or multivitamins in the last twelve months

The crude mean of plasma α-tocopherol concentration was slightly higher in cases compared to controls; plasma β-carotene concentration was similar in cases and controls. BMI was positively related to plasma α-tocopherol and negatively related to plasma β-carotene concentration. Pack-years of smoking and educational level were not related to plasma antioxidant levels. Thus BMI, age and gender were always included in all adjusted analysis.

**Table 2:** *Relation between respiratory symptoms and levels of plasma β-carotene*

| Plasma β-carotene | | Number of | | Unadjusted | Adjusted * | |
|---|---|---|---|---|---|---|
| Quintiles | Median§ | Cases | Controls | OR | OR | 95% CI |
| 1† | 0.14 | 108 | 99 | 1.0 | 1.0 | |
| 2 | 0.23 | 103 | 100 | 0.94 | 0.97 | 0.65-1.44 |
| 3 | 0.30 | 92 | 100 | 0.84 | 0.80 | 0.53-1.20 |
| 4 | 0.41 | 83 | 98 | 0.78 | 0.77 | 0.51-1.17 |
| 5 | 0.61 | 105 | 99 | 0.97 | 0.97 | 0.64-1.48 |
| Continuous ‡ | | 491 | 496 | 0.84 | 0.81 | 0.63-1.04 |

OR = prevalence Odds Ratio; 95% CI= 95% Confidence Interval

* adjusted for age, gender, BMI

† reference category

‡ presented for the difference between the 90th percentile (0.607 μmol/l) and the 10th percentile (0.138 μmol/l) of plasma concentration of β-carotene

§ median of plasma β-carotene concentration (μmol/l) in each quintile based on the controls

The adjusted Odds Ratios (ORs) of respiratory symptoms in 2nd to 5th quintiles of plasma β-carotene compared to reference were below one (table 2). The OR of respiratory symptoms in subjects with a high (0.607 μmol/l) versus a low (0.138 μmol/l) plasma concentration of β-carotene was borderline statistically significant (OR=0.81; 95%CI 0.63-1.04). The results were similar if asthma symptoms, chronic bronchitis symptoms or dyspnea were considered separately (table 3). After a more stringent definition of the cases and controls with $FEV_1$, the adjusted OR of a high versus a low β-carotene concentration decreased slightly (OR=0.65; 95%CI 0.37-1.14). The inverse relation between plasma β-carotene and case-control status tended to be slightly stronger in former smokers (OR=0.61; 95%CI 0.36-1.03) than in never smokers (OR=0.89; 95%CI 0.67-1.18) but the difference between former and never smokers was not statistically significant. Additionally adjustment for pack-years of smoking did not change the OR of respiratory symptoms for a high versus a low plasma β-carotene level in former smokers.

**Table 3:** *Prevalence Odds Ratios of asthma, chronic bronchitis-like symptoms and dyspnea for continuous levels of plasma β-carotene (n=496 controls)*

| Model | Asthma symptoms* n=180 | | Bronchitis symptoms† n=200 | | Dyspnea‡ n=111 | |
|---|---|---|---|---|---|---|
| | OR§ | 95% CI§ | OR§ | 95% CI§ | OR§ | 95% CI§ |
| Unadjusted | 0.80 | 0.60-1.14 | 0.85 | 0.63-1.15 | 0.88 | 0.61-1.27 |
| Age, gender, BMI adjusted | 0.87 | 0.62-1.21 | 0.82 | 0.59-1.14 | 0.80 | 0.53-1.21 |

* see the case definition in methods

† excluding subjects with asthma-like symptoms

‡ excluding subjects with asthmatic-like and chronic bronchitis-like symptoms

§ prevalence Odds Ratios (OR) with 95% confidence interval (95%CI) presented for the difference between the 90th percentile (0.607 μmol/l) and the 10th percentile (0.138 μmol/l) of plasma concentration of β-carotene

The adjusted ORs of respiratory symptoms in 2nd to 5th quintiles of plasma α-tocopherol compared to reference were around one (table 4). The adjusted OR of respiratory symptoms in subjects with a high (39.50 μmol/l) versus a low (20.42 μmol/l) plasma α-tocopherol was slightly elevated but not statistically significant.

**Table 4:** *Relation between respiratory symptoms and levels of plasma α-tocopherol*

| Plasma α-tocopherol | | Number of | | Unadjusted | Adjusted* | |
|---|---|---|---|---|---|---|
| Quintiles | Median§ | Cases | Controls | OR | OR | 95% CI |
| 1† | 20.3 | 91 | 97 | 1.0 | 1.0 | |
| 2 | 25.0 | 71 | 100 | 0.76 | 0.70 | 0.46-1.09 |
| 3 | 28.6 | 93 | 99 | 1.00 | 0.85 | 0.56-1.31 |
| 4 | 31.9 | 88 | 98 | 0.96 | 0.77 | 0.50-1.20 |
| 5 | 39.3 | 148 | 102 | 1.55 | 1.17 | 0.76-1.82 |
| Continuous‡ | | 491 | 496 | 1.65 | 1.36 | 0.98-1.89 |

OR=prevalence Odds Ratio; 95% CI= 95% Confidence Interval

* adjusted for age, gender, BMI

† reference category

‡ presented for the difference between the 90th percentile (39.50 μmol/l) and the 10th percentile (20.42 μmol/l) of plasma concentration of α-tocopherol

§ median of plasma α-tocopherol concentration (μmol/l) in each quintile based on the controls

Considering asthma, chronic bronchitis and dyspnea separately, the adjusted OR of plasma α-tocopherol for dyspnea was statistically significantly elevated (Table 5). Dyspnea is a common symptom in cardiovascular diseases. Risk factors for cardiovascular disease, such as, an elevated plasma total cholesterol level, diastolic blood pressure and a decreased concentration of plasma high density lipoprotein

cholesterol were associated with dyspnea but not with the other respiratory symptoms. Adjustment for these risk factors in the relation between plasma α-tocopherol and dyspnea did, however, not change the positive association (results not shown). After repeating the analysis excluding cases with dyspnea (n=111), the adjusted OR of respiratory symptoms for plasma α-tocopherol was not different from one (OR=1.24; 95% CI: 0.87-1.78).

**Table 5:** *Prevalence Odds Ratios of asthma, chronic bronchitis-like symptoms and dyspnea for continuous levels of plasma α-tocopherol (n=496 controls)*

| Model | Asthma symptoms* n=180 | | Bronchitis symptoms† n=200 | | Dyspnea‡ n=111 | |
|---|---|---|---|---|---|---|
| | OR§ | 95% CI§ | OR§ | 95% CI§ | OR§ | 95% CI§ |
| Unadjusted | 1.63 | 1.10-2.43 | 1.26 | 0.85-1.88 | 2.70 | 1.73-4.20 |
| Age, gender, BMI adjusted | 1.40 | 0.90-2.20 | 1.16 | 0.74-1.82 | 2.10 | 1.28-3.43 |

* see the case definition in methods

† excluding subjects with asthma-like symptoms

‡ excluding subjects with asthmatic-like and chronic bronchitis-like symptoms

§ prevalence Odds Ratios (OR) with 95% confidence interval (95%CI) presented for the difference between the 90th percentile (39.50 μmol/l) and the 10th percentile (20.42 μmol/l) of plasma concentration of α-tocopherol

Cases with asthma and chronic bronchitis symptoms used more diets for diabetes and for restriction on fat and cholesterol than controls. This could have lead to increased levels of plasma α-tocopherol in cases. Excluding all subjects with these diets (27 cases and 16 controls) did not essentially change the adjusted OR of plasma α-tocopherol (OR=1.19; 95%CI: 0.82-1.73). Using the more stringent case-control definition with $FEV_1$, the adjusted OR for plasma α-tocopherol increased slightly (OR=1.60; 95%CI 0.83-3.11) but the OR was not statistically significant. There was no essential difference between former (OR=1.15, 95% CI 0.61-2.18) and never smokers (OR=1.28, 95% CI 0.82-1.99) in the relation between plasma α-tocopherol and respiratory symptoms.

## Discussion

After adjustment for age, gender and BMI, cases tended to have lower plasma concentrations of β-carotene than controls but this association was not statistically significant at the 5% level. Plasma α-tocopherol concentration was slightly elevated in cases compared to controls. This was mainly due to the statistically significantly

elevated plasma α-tocopherol concentration in cases with dyspnea compared to controls.

Lower plasma β-carotene concentration in cases compared to controls could occur when plasma samples of cases were stored longer than controls. All plasma samples were not stored for longer than half a year at -20 °C. A stability study showed that this time period was short enough to prevent the loss of plasma concentration of α-tocopherol and β-carotene [109]. The average time and range for the plasma samples stored at -20 °C was comparable for cases (mean 111 days) and controls (mean 105 days). Therefore, the OR of plasma β-carotene for respiratory symptoms was not likely to be affected by storage time.

Our observation that cases had lower plasma β-carotene concentrations than controls is consistent with a recent cross-sectional study by Rautalahti and co-workers who showed a significant inverse relation between plasma β-carotene and COPD symptoms. However, this study was in a large group of male smokers (n=29,133) [19]. To our knowledge there have been no other results published on the relation between plasma β-carotene and respiratory symptoms.

Plasma levels of β-carotene could be a better marker of exposure in the lung tissue than the intake of β-carotene. Studies on dietary β-carotene did not find a significant association with the incidence of adult-onset asthma [17], the incidence of COPD [16] or the prevalence of several respiratory symptoms [93]. In contrast, plasma β-carotene was inversely related to the prevalence of respiratory symptoms in the study of Rautalahti and co-workers [19] and in the present study. Additionally, the Pearson's correlation coefficient between blood concentration of β-carotene and lung tissue (r=0.72) was higher than the correlations between dietary intake of β-carotene and the concentrations in lung tissue (r=0.54) [46].

Plasma α-tocopherol concentration was higher in cases with dyspnea than in controls. In the selection of cases, dyspnea was included as a symptom for emphysema but dyspnea is also a common symptom for cardiovascular disease. This was confirmed because cases with dyspnea, but not cases with asthma or chronic bronchitis-like symptoms, had higher prevalences of risk factors for cardiovascular disease, such as elevated plasma total cholesterol level and diastolic blood pressure. Adjustment for these risk factors did, however, not change the positive association between plasma α-tocopherol and dyspnea. So, the positive association between plasma α-tocopherol and dyspnea remains inexplicable.

In the present case-control study in non-smokers, plasma α-tocopherol was not associated with chronic bronchitis-like and asthmatic symptoms but it was positively associated with dyspnea. A recent cross-sectional investigation among smokers showed, however, an inverse association between serum levels of α-

tocopherol and COPD-symptoms, both chronic bronchitis symptoms and dyspnea. This study excluded all subjects who reported having symptoms of cardiovascular disease at baseline, such as angina pectoris and arrhythmias [19]. Since the present study was not able to identify these symptoms, cases with dyspnea might have had these cardiovascular disease symptoms. To our knowledge, no other studies on the relation between plasma α-tocopherol and chronic respiratory symptoms were published. Studies on dietary vitamin E as marker of exposure in relation to respiratory disease were inconsistent in their results [17 93]. However, plasma α-tocopherol was shown to be a better marker than dietary vitamin E as exposure in the lung tissue [46].

In conclusion, the present case-control study among non-smokers suggested that subjects with a high plasma level of β-carotene tended to have fewer chronic respiratory symptoms than subjects with a low plasma level of β-carotene. Plasma α-tocopherol was not associated with asthma and chronic bronchitis-like symptoms. Subjects with a high level of plasma α-tocopherol were more likely to have dyspnea than subjects with a low level of plasma α-tocopherol. It remains intruiging if α-tocopherol or other antioxidants are beneficial for respiratory symptoms in smokers who are exposed to relatively high oxidant levels.

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## Appendix

| Chronic Respiratory symptoms | Questions in the questionnaire |
|---|---|
| Cough | Do you cough during wintertime on most days for at least three months a year? |
| Phlegm | Do you bring up phlegm during wintertime on most days for at least three months a year? |
| Productive cough | Have you had productive cough for a period of three weeks in the last 3 years? |
| SOB when walking actively | Are you troubled by shortness of breath when hurrying on level ground or when walking at normal pace on a slope or flight of stairs? |
| SOB when walking compared to persons of the same age | Are you short of breath when walking with other people of your own age on level ground? |
| Wheeze with SOB | Has wheezing with shortness of breath, in the last twelve months troubled you? |
| Wheeze without a cold | Has wheezing, not due to a cold or the flu in the last twelve months troubled you? |
| Attacks of SOB | Are you being woken by attacks of shortness of breath in the last twelve months? |
| Ever asthma | Have you ever had asthma? |

SOB=Shortness of Breath

# CHAPTER 7

# Plasma concentrations of the antioxidants β-carotene and α-tocopherol in relation to lung function


## Abstract

*Background* The aim of this investigation was to study the relation between plasma antioxidants (β-carotene and α-tocopherol) and lung function in Dutch adults aged 20-59 years.

*Methods* A random sample (n=367) was drawn from the participants in a population based cross-sectional study in 1995. Linear regression analysis was performed with forced expiratory volume in one second ($FEV_1$) or forced vital capacity (FVC) as dependent variables and plasma antioxidant concentration as independent variable. We adjusted for age, height, gender, smoking status, pack-years of smoking, and alcohol consumption and we present the results as a difference between the 90th and 10th percentile of plasma antioxidant concentration.

*Results* Subjects with a high plasma β-carotene level tended to have a higher $FEV_1$ (73 ml, SEM 60 ml; p=0.22) and a higher FVC (147 ml, SEM 76 ml; p=0.052) than subjects with a low plasma β-carotene level. The difference was only observed in current and never smokers but not in former smokers. There was no difference in lung function between subjects with high and low plasma α-tocopherol concentrations.

*Conclusions* The results suggest that subjects with a high plasma β-carotene tended to have a higher FVC than subjects with a low plasma β-carotene concentration. The difference for $FEV_1$ between high and low levels of plasma β-carotene tended to be in the same positive direction as for FVC but this was not statistically significant. Plasma α-tocopherol was not associated with lung function.


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## Introduction

Antioxidants, such as vitamins C, E and β-carotene may beneficially affect lung function [96-98]. These antioxidants can scavenge endogenous and/or environmental oxidants in the lung and could, therefore, prevent a permanent loss of lung function over time [24].

Investigating the relation between blood levels of antioxidants and lung function is important to elucidate a possible association of antioxidants with respiratory disease because lung function is thought to be a more objective measurement than self-reported respiratory symptoms and blood levels of antioxidants are likely to be better markers of exposure in the lung tissue than dietary intake of antioxidants measured by questionnaire [46,48,107]. However, most studies so far have been investigating the relations between dietary antioxidants and lung function [13-15,40,93]. To our knowledge, there has only been one cross-sectional study (798 men) on blood levels of β-carotene in relation to lung function suggesting a beneficial effect [43]. Another study (83 men) did not show associations between blood levels of antioxidants and the development of airway obstruction ($FEV_1$<75%FVC) during five subsequent years [41]. Thus, very few studies have investigated blood levels of antioxidants (β-carotene and α-tocopherol) in relation to lung function.

Therefore, we studied the relation between the plasma antioxidants (β-carotene and α-tocopherol) and lung function in a random sample of Dutch adults.

## Methods

### *Study population*

A random sample was drawn from the MORGEN-study in 1995 (the monitoring project on risk factors and health in the Netherlands). This is a cross-sectional investigation on the prevalence of risk factors for chronic diseases in a randomly selected sample of the Dutch population aged 20 to 59 years in three towns in the Netherlands (Amsterdam, Doetinchem and Maastricht). The random sample was selected from a total of 4,238 subjects with plasma samples in 1995 stratified according to gender and age (four 10-years age categories) resulting in eight strata of equal size (n=56). In the resulting 448 subjects, plasma concentrations of α-tocopherol and β-carotene were determined.

### *Data collection*

All respondents from the MORGEN-study filled out a general and a semi-quantitative food frequency questionnaire and underwent a physical examination [86,87,93]. The general questionnaire provided information about demographic variables; life-style factors, such as smoking (smoking status and pack-years of smoking) and alcohol

consumption (non drinkers, light drinkers: >0-3 glasses a day, moderate drinkers: more than 3 glasses a day); environmental factors such as dampness of the house; respiratory symptoms (selected from the Dutch part of the European Community Respiratory Health Survey) [84,99] and the presence of other chronic diseases. The physical examination included measurements of height, weight and lung function. In addition, blood (non-fasting) was collected in all subjects for routinely measurements of plasma total cholesterol and glucose. Plasma antioxidants (α-tocopherol and β-carotene) were determined in the random sample.

Blood specimens were collected in ethylene diamine tetraacetic acid (EDTA) vacutainer tubes and stored in the refrigerator shielded from the light. Within four hours after collection, the blood was centrifuged to obtain plasma. Aliquots of plasma were stored at -20 °C for six months or less. After that, plasma samples were stored at -80 °C at the National Institute of Public Health and the Environment (RIVM). Concentrations of α-tocopherol and β-carotene in each subject were measured by reverse-phase high-performance liquid chromatography (HPLC) in one run. The method was modified as described by Hess and co-workers [75]. The coefficient of variation of the HPLC measurement was 4.1% for plasma β-carotene and 3.1% for plasma α-tocopherol. Due to reasons that occurred randomly, such as not having enough plasma or a defective HPLC device, plasma antioxidant levels could not be determined in nine subjects.

Lung function was measured with a heated pneumotachometer (Jaeger, Germany). Calibration took place twice a day. Trained paramedics performed the lung function measurements. Subjects were seated in an upright posture with a fixed mouthpiece, which was adjusted for the height of each individual; in addition, a nose clip was used. No lung function data was available for 34 subjects because of practical reasons that operated randomly, such as non-availability of lung function devices, time constraints, etc. All subjects who did not achieve at least three technically acceptable manoeuvres, such as manoeuvres with cough, a hesitant start or an early termination were excluded from analysis. Additionally, those subjects who could not perform two reproducible manoeuvres according to ERS 1993 criteria [77] were excluded from analyses. Analyses were based on the maximum value of the reproducible manoeuvres of $FEV_1$ and FVC.

## Data analysis

Models for $FEV_1$ and FVC were fitted with multiple linear regression [60]. We used the following basic adjusted model: $FEV_1$ or FVC divided by height squared as the dependent variable with age, age squared and gender as independent variables. This model was based on all subjects (n=6,555) with reproducible lung function

measurements during the years of 1994 and 1995 [93]. The shape of the relation between each plasma antioxidant and lung function was investigated by classifying the independent variables (plasma antioxidants) into quintiles of the total population. Since no essential deviation from linearity was observed, plasma antioxidants β-carotene and α-tocopherol were entered as continuous independent variables into the regression model. Regression coefficients (in ml) were calculated for a standard height of 1.70 meter and were expressed as the difference in $FEV_1$ or FVC between the $90^{th}$ percentile and the $10^{th}$ percentile of plasma antioxidant concentration.

Adjustment for plasma total cholesterol is common in studies with cardiovascular disease as an endpoint and plasma α-tocopherol as independent variable because plasma total cholesterol is potentially a confounding factor in this relation. However, plasma total cholesterol is not a known risk factor for lung disease. So, we did not adjust for plasma total cholesterol in the relation between plasma α-tocopherol and lung function.

We evaluated the effect of the following potential confounders in the relation between plasma antioxidants and lung function: pack-years of smoking, smoking status, educational level, body mass index (BMI), physical activity, dietary antioxidants (vitamins C, E and β-carotene) and alcohol consumption. We could not consider heavy alcohol consumption (>6 glasses/day) because of the small percentage (2%) present in this sample. In addition, we studied the presence of effect modification of smoking status on the relation between plasma antioxidants and lung function by stratified analysis.

## Results

Plasma levels of β-carotene and α-tocopherol could be determined in 439 out of the 448 subjects. Of those, 34 subjects did not perform a lung function measurement and six subjects had missing values for pack years of smoking. Of the remaining 399 subjects, 385 subjects (96%) had at least three technically acceptable lung function manoeuvres, of which 367 subjects had reproducible measurements for $FEV_1$ and 369 subjects had reproducible measurements for FVC.

Subjects who were excluded from analyses because they had not performed a lung function test (N=34) or if they did not meet acceptability and reproducibility criteria for $FEV_1$ (N=32) or FVC (N=30) were older, less physical active, had a lower educational level and higher body mass index (BMI) than those subjects who were included in the analysis (results not shown).

**Table 1:** *Mean (SD)* * *characteristics for the overall group and by smoking status*

| Characteristics | Overall group | Smoking status | | |
|---|---|---|---|---|
| | | Current | Former | Never |
| | (n=367) | (n=129) | (n=104) | (n=134) |
| Age (years) | 39.7 (11.7) | 40.3 (10.7) | 44.1 (10.7) | 35.6 (12.0) |
| Gender (% women) | 50.7 | 52.7 | 44.2 | 53.7 |
| Pack-years† | 15.4 (12.7) | 17.6 (12.9) | 12.7 (11.8) | - |
| Educational level (%)‡ | | | | |
| Low | 42.1 | 48.1 | 41.7 | 36.6 |
| Intermediate | 34.2 | 31.0 | 36.9 | 35.1 |
| High | 23.8 | 20.9 | 21.4 | 28.4 |
| BMI ($kg/m^2$) | 24.7 (3.8) | 24.4 (3.8) | 25.6 (3.8) | 24.4 (3.8) |
| Physical Activity (%) | 69.9 | 58.1 | 75.7 | 76.9 |
| Alcohol consumption | | | | |
| none | 39.5 | 37.2 | 33.7 | 46.3 |
| low (>0-3 glass/day) | 47.7 | 43.4 | 53.8 | 47.0 |
| moderate( >3 glass/day) | 12.8 | 19.4 | 12.5 | 6.7 |
| Supplement use (%)§ | 11.7 | 7.8 | 15.4 | 12.7 |
| Respiratory symptoms (%)‖ | 39.5 | 48.8 | 40.6 | 29.9 |

* for those subjects with technically acceptable and reproducible $FEV_1$ manoeuvres

† one pack-year is equal to smoking 20 cigarettes a day for one year

‡ low (intermediate secondary education or less), intermediate (intermediate vocational or higher secondary education) and high educational level (higher vocational or university education)

§ daily vitamin supplement use of vitamin A, C, E and/or multivitamins in the last twelve months

‖ one or more of the following respiratory symptoms: cough, phlegm, productive cough, wheeze, shortness of breath, nocturnal attacks of shortness of breath, ever asthma

Table 1 shows the characteristics of the study population by smoking status. Current smokers were less physically active compared to former and never smokers. Further, current smokers included more moderate alcohol drinkers and subjects with respiratory symptoms than never smokers. Former smokers were older and had a higher BMI than current and never smokers. In addition, former smokers included fewer non-alcohol drinkers compared to never smokers.

Table 2 presents the mean lung function adjusted for age, height and gender and the crude plasma antioxidant levels by smoking status. Current smokers had lower plasma β-carotene levels than former and never smokers. Former smokers had a higher mean $FEV_1$, FVC and plasma level of α-tocopherol.

Adjustment for educational level, BMI and physical activity did not essentially change the regression coefficients of plasma antioxidants on lung function. These variables were, therefore, not included in the adjusted models.

**Table 2:** *Mean (SD) lung function and plasma levels of antioxidants*

| Variables | Overall group | Smoking status | | |
|---|---|---|---|---|
| | | Current | Former | Never |
| Number of subjects * | 367 | 129 | 104 | 134 |
| Lung function (L) † | | | | |
| $FEV_1$ | 3.55 | 3.46 | 3.66 | 3.57 |
| FVC | 4.49 | 4.46 | 4.64 | 4.40 |
| Plasma levels (μmol/l) | | | | |
| β-carotene | 0.31 (0.19) | 0.28 (0.16) | 0.34 (0.21) | 0.33 (0.19) |
| α-tocopherol | 30.4 (9.5) | 30.2 (10.0) | 32.3 (9.4) | 29.0 (8.8) |

* These numbers are for subjects with technically acceptable and reproducible $FEV_1$ manoeuvres; for technically acceptable and reproducible FVC manoeuvres these numbers were respectively 369, 123, 108 and 138

† Lung function was adjusted for age, height and gender by regression analysis (see methods) and presented for a standard height of 1.70 m

Smoking status and pack-years of smoking were both associated with lung function and with plasma antioxidant concentrations (results not shown). Therefore, smoking status and pack-years of smoking were always included in all adjusted models. Moderate alcohol consumption was associated with a lower plasma β-carotene concentration and a higher $FEV_1$ and FVC compared to no alcohol consumption. This elevated lung function with moderate alcohol consumption was observed only in current and never smokers. Moderate alcohol consumption was not associated with plasma α-tocopherol concentration (results not shown). Alcohol consumption (light or moderate versus none) was additionally included as a confounder in the final model on plasma β-carotene and lung function.

Table 3 presents the relations between plasma β-carotene and lung function. After adjustment for age, height, gender, smoking and alcohol consumption in the group as a whole, subjects with a high plasma β-carotene level had a slightly higher $FEV_1$ but this was not statistically significant compared to subjects with a low plasma level of β-carotene. The age, height and gender adjusted difference in $FEV_1$ between subjects with a high and a low plasma β-carotene level was borderline statistically significant (mean difference 107 ml, SEM 60 ml; p=0.074). The magnitude of this difference decreased by more than a half (52 ml) after adjustment for pack-years of smoking and increased slightly (73 ml) after additional adjustment for alcohol consumption. The difference in $FEV_1$ between subjects with a high and a low plasma β-carotene level was observed in current and never smokers but not in former smokers. After adjustment for age, height, gender, smoking and alcohol consumption in the group as a whole, subjects with a high plasma β-carotene concentration had a borderline significantly higher FVC (147 ml, SEM 76 ml; p=0.052) compared to

subjects with low levels of plasma β-carotene. The magnitude of this difference (147 ml) was similar to the magnitude of the difference in FVC (134 ml) between subjects with a high and a low plasma β-carotene after adjustment for only age, height and gender but the latter association was statistically significant. This difference in FVC between high and low levels of plasma β-carotene was observed only in current and never smokers.

Table 4 shows that in the group as a whole, the adjusted difference for $FEV_1$ and FVC between high and low plasma α-tocopherol concentration was not different from zero. There were no significant differences between the smoking categories, although the differences were again in the direction of a higher lung function with higher plasma α-tocopherol levels for current and never smokers but not for former smokers.

Adjustment for dietary antioxidants (vitamins C, E and β-carotene) did not essentially change the relations between plasma antioxidants and lung function (results not shown).

## Discussion

Subjects with a high plasma β-carotene concentration had a higher FVC compared to subjects with a low plasma β-carotene concentration which was borderline statistically significant. A higher $FEV_1$ for a high versus a low plasma β-carotene level tended to be observed but this was not statistically significant. These differences in lung function between subjects with high and low levels of plasma β-carotene were observed in current and never smokers but not in former smokers. Subjects with a high plasma α-tocopherol level did not have a higher lung function than subjects with low levels of plasma α-tocopherol.

Selection bias may have occurred in this random sample because subjects who did not have a lung function measurement (n=34) or did not fulfil ERS criteria (n=32) were excluded from analysis. However, the reason for not having a lung function measurement was practical and occurred randomly because of non-availability of lung function devices and time constraints suggesting that lung function was not likely to be different. In addition, plasma levels of antioxidants were also not different in subjects with or without lung function measurements, so selection bias did probably not occur in this group. Subjects who did not fulfil ERS criteria were estimated to have a lower lung function [110].

**Table 3:** *The relation[||] between plasma β-carotene and lung function for the overall group and by smoking status*

| Lung function | Overall group | | Smoking status | | | | | |
|---|---|---|---|---|---|---|---|---|
| Adjustments | | | Current | | Former | | Never | |
| $FEV_1$ | n=367 | | n=129 | | n=104 | | n=134 | |
| | β | (SEM) | β | (SEM) | β | (SEM) | β | (SEM) |
| Age, height & gender (basic) [†] | 107.0 | (59.8) | 195.0 | (116.4) | -5.1 | (99.5) | 66.1 | (101.4) |
| Basic + smoking [‡] | 51.9 | (60.0) | 163.8 | (122.0) | -19.4 | (97.4) | 66.1 | (101.4) |
| Basic + smoking + alcohol [§] | 73.0 | (59.9) | 184.8 | (119.3) | -0.6 | (100.7) | 91.3 | (102.7) |
| FVC | n=369 | | n=123 | | n=108 | | n=138 | |
| | β | (SEM) | β | (SEM) | β | (SEM) | β | (SEM) |
| Age, height & gender (basic) [†] | 133.7 | (75.2)* | 187.8 | (148.1) | 6.3 | (137.5) | 189.3 | (114.8) |
| Basic + smoking [‡] | 124.7 | (75.8) | 210.3 | (155.7) | 4.0 | (138.3) | 189.3 | (114.8) |
| Basic + smoking + alcohol [§] | 147.4 | (75.6) | 217.7 | (153.1) | 21.3 | (142.9) | 217.4 | (115.5) |

* $p < 0.05$

[†] see methods for exact adjustment

[‡] smoking adjustment: pack-years of smoking (zero for never smokers) and smoking status (only for overall group)

[§] alcohol adjustment: alcohol consumption (dummies low or moderate versus none)

[||] regression coefficients are presented as a difference in $FEV_1$ and FVC (in ml for a height of 1.70 m) between subjects in the $90^{th}$ percentile (0.57 $\mu mol/l^{-1}$) and those in the $10^{th}$ percentile (0.11 $\mu mol/l^{-1}$) of plasma β-carotene concentration

**Table 4:** *The relation [§] between plasma α-tocopherol and lung function for the overall group and by smoking status*

| Lung function | Overall group | | Smoking status | | | | | |
|---|---|---|---|---|---|---|---|---|
| Adjustments | | | Current | | Former | | Never | |
| $FEV_1$ | n=367 | | n=129 | | n=104 | | n=134 | |
| | β | (SEM) | β | (SEM) | β | (SEM) | β | (SEM) |
| Age, height & gender (basic) [†] | 21.4 | (60.5) | 92.8 | (93.6) | -125.7 | (107.3) | 56.6 | (112.7) |
| Basic + smoking [‡] | 17.0 | (58.9) | 88.8 | (93.5) | -108.4 | (105.3) | 56.6 | (112.7) |
| FVC | n=369 | | n=123 | | n=108 | | n=138 | |
| | β | (SEM) | β | (SEM) | β | (SEM) | β | (SEM) |
| Age, height & gender (basic) [†] | 29.7 | (76.4) | 39.3 | (120.3) | -109.8 | (148.8) | 139.8 | (129.1) |
| Basic + smoking [‡] | 25.0 | (75.5) | 39.3 | (120.8) | -107.2 | (149.7) | 139.8 | (129.1) |

[†] see methods for exact adjustment

[‡] smoking adjustment: pack-years of smoking (zero for never smokers) and smoking status (only for overall group)

[§] regression coefficients are presented as a difference in $FEV_1$ and FVC (in ml for a height of 1.70 m) between subjects in the 90$^{th}$ percentile (42.3 μmol/l$^{-1}$) and those in the 10$^{th}$ percentile (20.2 μmol/l$^{-1}$) of plasma α-tocopherol concentration

Plasma antioxidant levels were, however, not consistently different in subjects who were excluded or included in the analysis. So if these subjects had been included in the analysis, the difference in lung function for a high versus a low antioxidant level would be slightly lower. However, these excluded subjects were only a small percentage (8%) of the total study population and the estimated lower lung function was small with 5% [110]. Therefore, selection bias probably did not have an influential impact on the results of the present study.

Smoking is known to be a confounder in the relation between $FEV_1$ [111] and plasma β-carotene [43,108]. In this sample, the borderline significant association between plasma β-carotene and $FEV_1$ did not remain after adjustment for pack-years of smoking. A similar impact of adjustment for pack-years of smoking on the relation between plasma β-carotene and $FEV_1$ was observed in the study of Chuwers et al [43].

Alcohol consumption appeared to be a confounder in the relation between plasma β-carotene and lung function, in particular, in current and never smokers. We observed that moderate alcohol consumption versus no alcohol consumption was associated with a higher $FEV_1$ and FVC. Two other studies suggested that a moderate intake of alcohol compared to a low intake was associated with higher FVC [112] and that smokers who were consuming moderate alcohol had a better lung function [113]. Further, the present study showed that the concentration of plasma β-carotene was lower in subjects with moderate alcohol consumption. This was also observed in other studies [114,115]. So studies investigating the association between plasma β-carotene and lung function that do not adjust for alcohol consumption may underestimate this association if alcohol consumption is associated with lung function, which is still a point of discussion in the literature.

A high versus a low plasma concentration of β-carotene may be related to a higher lung function if the storage time of the plasma at –20 °C was shorter in those subjects with a higher lung function. A stability study showed that plasma concentrations of β-carotene and α-tocopherol were not affected by storage at -20 °C up to 180 days [109]. The mean storage time at -20 °C of about half of our plasma samples was 80 days (range 14 to 162 days). The other samples were directly stored at -80 °C. In addition, storage time at –20 °C was not essentially different for subjects with a high versus a low lung function suggesting that our results on plasma β-carotene and lung function were not attenuated by storage time.

In the present study, a high versus a low plasma β-carotene concentration of 0.46 μmol/l (90th versus 10th percentile) was associated with a non-significantly higher $FEV_1$ of 73 ml and a borderline significantly higher FVC of 147 ml. The observed difference in $FEV_1$ for a high versus low plasma β-carotene level may be clinically relevant because it was about equivalent to three times the annual decline

of $FEV_1$ (25 ml) in non-smoking Dutch adults [116]. Additionally, it was approximately equivalent to the adverse effects of five years of heavy smoking (≥25 cigarettes per day) on $FEV_1$ decline in Dutch adults [117].

The magnitude of the association between plasma β-carotene and lung function in our study can be compared with one other cross-sectional study among 798 men who had been exposed to asbestos and were mostly former (63%) and current smokers (22%) [43]. In that study, a difference in the concentration of serum β-carotene of 0.29 μmol/l was significantly associated with a 90 ml higher $FEV_1$ and an 82 ml higher FVC [43]. A difference of 0.29 μmol/l plasma β-carotene in our study would be associated with a 46 ml higher $FEV_1$ and a 93 ml higher FVC. So, the difference in FVC for a high versus a low plasma β-carotene level but not the difference in $FEV_1$ was comparable with Chuwers et al [43].

The present study did not show an association between plasma α-tocopherol and lung function. These results are consistent with one small follow-up study (83 men); serum levels of α-tocopherol were not associated with the development of airway obstruction ($FEV_1 \leq 75\%$ FVC) during five subsequent year's [41]. So far, there is no evidence that α-tocopherol levels in blood are related to lung function.

A higher lung function tended to be observed for high versus low plasma antioxidant levels only in current and never smokers and not in former smokers but the difference between the smoking groups was not statistically significant. The lack of a statistically significant difference between the smoking groups may have resulted from a lack of statistical power. Another larger study (n=798) also observed in separate analyses that a possible beneficial effect of serum β-carotene on lung function was smaller in former smokers compared to never and current smokers [43]. The authors did, however, not give a possible explanation for the difference between the smoking groups. In the present study, former smokers were different from current and never smokers in a number of characteristics. Adjustment for all these known factors (such as age, body mass index, and alcohol consumption) did not change the observed relations in former smokers. Although the mean levels of lung function and plasma α-tocopherol were higher in former smokers compared to current and never smokers, we have no clear explanation for the observed difference of the relation between plasma antioxidants and lung function in former smokers.

In conclusion, the results of the present study suggest that subjects with a high plasma β-carotene concentration tended to have a higher FVC than subjects with a low plasma β-carotene concentration. The difference in $FEV_1$ between those subjects with high and low plasma β-carotene levels tended to be in the same positive direction as for FVC but the difference was not statistically significant. Plasma α-tocopherol concentration was not associated with lung function.

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# CHAPTER 8

## General Discussion

## Introduction

This thesis addressed two main research questions. In the first part, we investigated whether antioxidants (in plasma, diet and supplement form) could modulate the acute respiratory effects of air pollution in three different studies. In the second part, we studied possible relations between antioxidants (in plasma and diet) and indicators of asthma and COPD, such as respiratory symptoms and lung function in three different population-based samples. The main findings of these studies are discussed below. Additionally, some methodological considerations of the studies are addressed and the results are compared quantitatively with other studies. Finally, the biological plausibility of the findings is discussed.

## Main findings

### *Antioxidants in relation to the acute respiratory effects of air pollution*

Two intervention studies investigated a possible modulation of the acute respiratory effects of ozone by antioxidant supplementation. In addition a panel study examined a possible modulation of the acute respiratory effects of winter air pollution by antioxidants in diet and serum. The results are summarised in table 1.

**Table 1:** *Results of the studies presented in this thesis on modulation on acute effects of air pollution by antioxidants*

| Ch. | Study design | Main pollutant | Suppl. | Antioxidants | Outcome | Beneficial association |
|---|---|---|---|---|---|---|
| 2 | Interv. | ozone | yes | vitamins C, E, | $FEV_1$, FVC, PEF | + |
| | | | | β-carotene | MMEF | - |
| 3 | Interv. | ozone | yes | vitamins C, E | $FEV_1$, FVC | + |
| | | | | | MMEF, PEF | - |
| 4 | Panel | PM10, BS, | no | diet vitamin C | PEF, resp. symp. | - |
| | | $NO_2$, $SO_2$ | | diet β-carotene | PEF, resp. symp. | - |
| | | | | plasma β-carotene | PEF | + |
| | | | | | resp. symp. | - |
| | | | | diet & plasma vit E | PEF, resp. symp. | - |

Ch. = Chapter number; Interv. = Intervention studies; BS = black smoke; Suppl. = supplementation
Resp. symp.: respiratory symptoms and medication use;
Associations: + = beneficial association; - = no association

The first intervention study in 1994 was a pilot study among 26 cyclists who performed lung function measurements (192 observations) before and after

exercise. Half of the group was randomly assigned to a daily antioxidant supplementation of vitamins C, E and β-carotene and the other half did not receive a placebo. The average 8-hour mean ozone concentration prior to post-exercise lung function measurement was 101 $\mu g/m^3$ (range 30-205 $\mu g/m^3$). This study suggested that there was an effect of ozone on $FEV_1$, FVC and PEF in the control group. There was no change in lung function after ozone exposure in the supplementation group. The difference in ozone effect between the groups was statistically significant for $FEV_1$, FVC and PEF. For comparison of the results of chapter 2 and 3, the regression coefficients of $FEV_1$ and FVC were presented in four different ways in the respective appendices. The results of the regression coefficients of FVC on ozone tended to be similar for the different ways of presentation: median, unweighted mean, mean weighted for inverse of the variance and mean weighted for the number of measurements of each subject. However, the ozone effect was not significant for the control group and for the difference in ozone effects between the study groups (appendix chapter 2). The results of the median and the weighted (for inverse of variance) mean regression coefficients of $FEV_1$ on ozone were similar but not for the unweighted mean and for the mean weighted for number of measurements. So, it appeared that in individual regression analysis the interpretation of the results is slightly dependent on the presentation of the regression coefficients.

We repeated the study in the summer of 1996 with a similar design but this time the study was placebo-controlled. In this study, 38 subjects (380 lung function measurements) participated until the end of the study and the antioxidant supplementation consisted of a cocktail of vitamins C and E. The average 8-hour mean ozone concentration prior to post-exercise lung function measurements was 86 $\mu g/m^3$ (range 32-199 $\mu g/m^3$). The table in the appendix of chapter 3 suggests that the interpretation of the results did not depend on different presentations of the regression coefficients. The results suggest a significant effect of ozone in the control group but not in the vitamin group. The difference in ozone effect between the groups was statistically significant for the mean regression coefficients and for the mean regression coefficients weighted for the inverse of the variance. So after the comparison of the results of chapter 2 and 3, it appeared that in particular in small groups (chapter 2), the results become sensitive to alternative ways of presenting the group mean effect.

In conclusion, both intervention studies suggest that antioxidant vitamin supplementation reduces the acute effect of ambient ozone on lung function, which is in accordance with the specified hypothesis in chapter 1.

We performed a panel study investigating a possible modulation of acute respiratory effects of winter air pollution by antioxidant intake or status (Chapter 4). If both panels (with and without chronic respiratory symptoms) were taken together, there was no clear overall respiratory effect of air pollution. In the panel with chronic respiratory symptoms, positive associations among PM10, black smoke, the prevalence of large PEF decrements and upper respiratory symptoms were observed. The results suggest that subjects with low levels of plasma β-carotene showed an effect of air pollution, in particular, for PM10 and black smoke in relation to large PEF decrements, whereas subjects with high levels of plasma β-carotene did not show an effect of air pollution. No difference in acute respiratory effects of air pollution was observed for a high versus a low dietary intake of vitamin C, E and β-carotene and for plasma α-tocopherol.

In conclusion, the results suggest that the acute respiratory effects of winter air pollution are reduced in subjects with high serum levels of β-carotene, which is partly in line with the specified hypothesis in chapter 1.

***Antioxidants in relation to indicators of asthma and COPD***

In this part of the thesis, we investigated whether antioxidants were associated with the prevalence of the indicators of asthma and COPD in the MORGEN study. This study is a cross-sectional investigation on the prevalence of risk factors for chronic diseases using self-administered questionnaires and a physical examination in a randomly selected sample of the Dutch population. The results are summarised in Table 2.

*Dietary antioxidants*

We examined first the relations between dietary antioxidants (vitamins C, E and β-carotene) and the prevalence of a number of respiratory symptoms and lung function in a population based sample in 6,555 adults (Chapter 5).

Our results suggested that a high dietary vitamin C and β-carotene intake was associated with a higher $FEV_1$ and FVC. Dietary vitamin E was not associated with lung function. None of the dietary antioxidants were consistently associated with the prevalence of a number of respiratory symptoms. So these results are only partly in accordance with the hypothesis

(chapter 1), that is, subjects with a high dietary intake of vitamin C and β-carotene had a higher lung function compared to subjects with a low dietary intake of these antioxidants.

**Table 2:** *Relations between antioxidants and respiratory symptoms or lung function*

| Ch. | Main endpoint | Source | Antioxidants | Association | | |
|---|---|---|---|---|---|---|
| | | | | beneficial | none | adverse |
| 5 | respiratory symptoms | diet | vitamins C, E, β-carotene | | vitamins C, E, β-carotene | |
| 5 | lung function | diet | vitamins C, E, β-carotene | vitamin C, β-carotene | vitamin E | |
| 6 | respiratory symptoms | plasma | α-tocopherol, β-carotene | β-carotene (borderline) | α-tocopherol | α-tocopherol & dyspnea |
| 7 | lung function | plasma | α-tocopherol, β-carotene | β-carotene & FVC (borderline) tendency $FEV_1$ | α-tocopherol | |

Ch.: Chapter number; borderline: borderline significant p <0.10;
tendency: same positive direction, but not statistically significant

*Plasma levels of antioxidants*

Since blood levels of antioxidants are likely to be better markers of the antioxidant exposure in the lung, we investigated the relation between plasma levels of antioxidants and respiratory symptoms or lung function as well.

First, we examined the relation between plasma levels of β-carotene or α-tocopherol and respiratory symptoms in a case-control sample of never and long-term former smokers who were selected from the MORGEN study. Our results suggested that cases (subjects with one or more chronic respiratory symptoms) tended to have lower plasma β-carotene levels than controls. Plasma α-tocopherol was not associated with asthma and chronic bronchitis symptoms but was positively associated with dyspnea. This adverse association of plasma α-tocopherol could not be explained by adjustment for cardiovascular risk factors and remains puzzling.

Second, we evaluated the relation between plasma antioxidants (β-carotene and α-tocopherol) and lung function in a random sample (n=367) of the MORGEN study. We found that subjects with a high plasma β-carotene concentration tended to have a higher FVC and $FEV_1$ than subjects with a low plasma β-carotene concentration but this was not statistically significant for $FEV_1$. Plasma α-tocopherol was not associated with lung function.

These results are only for plasma β-carotene in line with the hypothesis specified in chapter 1.

## Methodological considerations

### *Misclassification of exposure*

*Intake of antioxidants*

Most large epidemiological studies have been using self-administered food frequency questionnaires as a measure of dietary intake because of limited resources in relation to the large number of participating subjects. These questionnaires introduce inherent measurement errors, in particular, for micronutrients such as antioxidant (pro)-vitamins. The measurement error occurs because the questionnaires rely on the memory of the subjects, the stability of the dietary pattern and because of converting the data on foods into nutrients using food composition tables. The errors in the food composition tables are probably larger for antioxidant (pro)-vitamins than for macronutrients because the variation in the vitamin content in the foods is generally greater than the variation in macronutrient content [92].

The food frequency questionnaire used in this thesis (chapter 4 and 5) was developed to measure the habitual consumption of 178 food items during the last 12 months [86,87]. The reproducibility for estimated antioxidant intake with this food frequency questionnaire was fairly good, in particular for men as measured at six and 12 months intervals. The Pearson's correlation coefficient at 12 months for vitamin C was 0.74 for men and 0.70 for women; for vitamin E 0.73 for men and 0.67 for women; for β-carotene 0.78 for men and 0.59 for women [87].

*Plasma levels of antioxidants*

Vitamin C

The reproducibility of vitamin C in blood over time was measured within a validation study of a food frequency questionnaire. In this study, blood was drawn in January or February and in June or July (6 months in between). The correlation coefficient was low (r=0.29) [118].

Plasma vitamin C does not reflect the usual individual intake. These levels show a S-shaped curve with the intake of vitamin C per day [50]; an intake below 20 mg/day is not available for circulation but enters the cells immediately [79]; at a daily intake of 30 to 60 mg the curve is steepest and with a daily intake of 90 to 150 mg it reaches a plateau which coincides with a

plasma level of 1.2 to 1.5 mg/dl [34]. Daily intakes above 100 mg will partly be excreted in the urine [34,79]. So plasma vitamin C levels are controlled by a homeostatic mechanism. This implies that the relation between vitamin C intake and blood levels is not linear [48]. The cyclists in our second intervention study (Chapter 3) had baseline plasma vitamin C levels on average of 1.5 mg/dl (85 μmol/l) which is already in the plateau-phase of the curve. In conclusion, plasma vitamin C was not a good marker for dietary or supplemental intake of vitamin C. The storage of vitamin C is mostly within the cells, such as leukocytes. Dietary or supplemental intakes above 100 mg have been shown to increase the concentration in these cells [50].

Vitamin E

The reproducibility over time for blood vitamin E was estimated from a validation study on this food frequency questionnaire [87]. In this study blood samples were drawn at 4, 6 and 9 months. The reproducibility for levels of blood vitamin E was good with a small range in correlation coefficients from 4 to 9 months (0.81 to 0.77) [personal communication, M.C. Ocké, RIVM, the Netherlands]. Plasma levels of α-tocopherol are moderately related to vitamin E supplements [48]. We found that a daily supplementation of 75 mg of α-tocopherol for 10 weeks increased plasma α-tocopherol levels by 38% (chapter 2). A daily supplementation of 100 mg α-tocopherol for 15 weeks increased plasma levels by 48% (chapter 3).

β-Carotene

Reproducibility over time for blood levels of β-carotene was also calculated from a validation study [87]. The reproducibility was fairly good with a small range in correlation coefficients from 4 to 9 months (0.75 to 0.64) [personal communication, M.C. Ocké, RIVM, the Netherlands].

Blood levels of β-carotene are very sensitive to supplemental intake as they are not closely regulated by a homeostatic mechanism [48]. We showed that a daily supplement of 15 mg for 10 weeks increased the levels of plasma β-carotene by 150% (chapter 2).

*Relative validity of antioxidant intake with plasma antioxidant levels*

Most epidemiological studies use plasma levels of antioxidants (α-tocopherol and β-carotene) as objective markers for dietary intake. However, the correlations between dietary and plasma α-tocopherol and dietary and plasma β-carotene are often low. One possible explanation was already

mentioned, namely the inadequacy of the food frequency questionnaire to measure micronutrients correctly.

Another explanation for a weak correlation between vitamin E intake and α-tocopherol in blood is that the absorption of vitamin E is incomplete and variable (20-80% in various studies [34]). Additionally, the efficiency of absorption declines with increasing dose or amount per meal [79]. The correlation between levels of α-tocopherol in plasma and diet from a validation study developed for the used food frequency questionnaire in this thesis was 0.23 for men and 0.15 for women [87]. These correlation coefficients are low but fit well within the range of correlation coefficients for non-supplement users found for similar questionnaires [104-106,114,119].

An explanation for low correlations between plasma en dietary levels of β-carotene is that plasma levels of β-carotene depend on the rate of intestinal absorption, the efficiency of the enzymatic transformation into retinoids and the rate of clearance from plasma [79]. The above mentioned validation study showed low correlation coefficients (negative for men and 0.13 for women) between dietary intake measured with the food frequency questionnaire and plasma levels of β-carotene [87]. These correlation coefficients are lower compared to other validation studies [104,114,119] suggesting that our food frequency questionnaire is limited with respect to measuring the intake of β-carotene. A few factors may explain this. First, the relative validity of the vegetable intake, which is the main contributor of the β-carotene intake, was poor [86]. Second, some food items in the questionnaire, such as 'soup' and 'cabbage & kale' were heterogeneous with respect to β-carotene composition [86,87]. Third, the major sources of β-carotene in the Netherlands are yellow/orange and green vegetables and the bioavailability of β-carotene in these vegetables is low [34]. Furthermore, Dutch regulations do not allow β-carotene fortifications of foods, which implies that many different foods each contributed a little to the total intake of β-carotene [87].

Since the food frequency questionnaire was probably poor in classifying β-carotene intake, the associations between dietary β-carotene and respiratory symptoms or lung function in this thesis (chapter 4 & 5) may have been biased towards the null due to non-differential misclassification of exposure.

So, plasma levels of α-tocopherol and β-carotene are not a good marker for dietary intake of these antioxidants determined with our food

frequency questionnaire because of the occurring measurement errors in the food frequency questionnaire and the metabolic processes in the body.

*Levels of antioxidants in the lungs*

Plasma levels of antioxidants could be good markers for the level of antioxidants in the lungs. All three antioxidants (vitamin C, E and β-carotene) are present in the lungs, namely in the lung lining fluids and/or in the cells. The effect of supplementation of antioxidants on the concentration in the lung lining fluids has recently been investigated [120]. Twenty-four healthy non-smoking adults were daily supplemented with 500 mg vitamin C and 400 mg α-tocopherol for 7 days and the change in the levels of vitamin C and α-tocopherol in plasma and in the epithelial lining fluids were determined. Preliminary results suggest that plasma levels of vitamin C and α-tocopherol significantly increased in the supplementation group but not in the placebo group. However, the levels of these antioxidants in nasal lavage, bronchial lavage, and bronchoalveolar lavage did not change in both groups [120]. It should be noted that all subjects had levels of plasma vitamin C well below the plateau level and that the period of supplementation was very short. So a longer period of supplementation, such as, 10 or 15 weeks which we used in our intervention studies might still increase the concentration of antioxidants in the lung lining fluids. In addition, an increase of vitamin E in the lung epithelial cells rather than in the fluids can not be excluded in this study.

Plasma levels of antioxidants (β-carotene and α-tocopherol) are probably better markers of exposure in the lungs than dietary intake of antioxidants. To our knowledge there has only been one study correlating the concentrations of antioxidants in the lung tissue, blood levels and dietary intake [46]. In this study, 21 subjects underwent an open lung biopsy in which tissue samples were obtained. Blood samples were additionally drawn. Bronchoalveolar lavage (BAL) samples were only obtained in 12 subjects and β-carotene was often not detectable in BAL, so we omit this in the comparisons. In addition, all subjects filled in a semi-quantitative food frequency questionnaire with additional questions on vitamin supplementation (9 of the 21 subjects used vitamin supplements). Pearson's correlation coefficients were calculated between these three media for each antioxidant (see table 3).

**Table 3:** *Pearson's correlation coefficients between intake (both dietary and supplemental), serum and lung tissue antioxidant levels*

| | Intake | Serum |
|---|---|---|
| β-carotene | | |
| Serum | 0.26 | - |
| Lung tissue | 0.19 | 0.72 |
| α-tocopherol | | |
| Serum | 0.71 | - |
| Lung tissue | 0.20 | 0.47 |

Modified from Redlich et al [46]

As was hypothesised, the levels of antioxidants in blood appeared to be better correlated with the levels in the lung tissue than the intake of these antioxidants. There are, however, a few limitations to this study. First, the number of subjects was very small for a validation study. Second, only one blood sample was obtained, whereas three separate tissue samples from different parts of the lung were obtained. Third, no adjustments were made for total cholesterol levels which could have resulted in the spurious high correlation coefficient (r=0.71) between dietary vitamin E and plasma levels of α-tocopherol compared to other studies [87,106,119].

In conclusion, plasma or serum levels of antioxidants are better markers of exposure in the lungs than dietary intake measured by questionnaire.

***Selection bias***

In the first part of this thesis, selection bias could not have affected the results because subjects in intervention studies and in panel studies were serving as their own control in the data analysis.

In the second part of this thesis, we used the data of the MORGEN-study, which is an observational study, and selection bias could have been present. Selection bias might have occurred studying the relations between antioxidants and lung function (chapter 5 & 7) because subjects who did not meet ERS criteria for lung function measurements were excluded from analysis on antioxidants and lung function. If selection bias was present, our observed association between lung function and antioxidants would be slightly lower when these subjects were included in the analysis (see discussion sections of chapter 5 & 7).

***Confounding bias***

Confounding bias occurs when the observed association between exposure and effect differs from the true association because of a third variable that is correlated with the exposure variable and the health outcome. This third variable has to be an independent risk factor of the health outcome [121].

In the first part of this thesis, the characteristics of the subjects could not have been confounding variables since each subjects was his/her own control in the analysis. In addition, the two intervention studies were randomised so known and unknown confounding characteristics were probably evenly distributed over the groups.

In the second part of this thesis, several subject characteristics were known to be risk factors for respiratory symptoms (chapter 5 & 6) and lung function (chapter 5 & 7), such as age, gender, and smoking. These risk factors were also associated with the antioxidant intake or plasma. So we adjusted for these known confounding factors in the relations between antioxidants and respiratory symptoms or lung function. In conclusion, known confounding factors were taken into account. We can, however, not exclude that due to measurement error in the confounders some residual bias might have occurred in the observed associations.

***Study design***

In the second part of this thesis, the relations between antioxidants and the indicators of asthma and COPD were investigated cross-sectionally. In chapter 5, we did not find a relation between dietary antioxidants and respiratory symptoms. With such a design, it is possible that subjects altered their diet as a result of their symptoms and a potentially protective effect could have been biased towards the null hypothesis. It should be noted, however, that scientific evidence is scarce on the possible relation between antioxidants and respiratory disease and that, therefore, the general public is probably not aware of such a possible relation.

Another problem with this study design is that the exposure data (antioxidants) are simultaneously ascertained with the disease information (respiratory symptoms or lung function) [121]. The previous exposure to antioxidants of subjects might be more relevant because a time interval of several years is expected (lag time) between exposure and the onset of the chronic effects, such as, increased respiratory symptoms or a decreased lung

function. However, so far the precise etiological relevant time period until the occurrence of asthma and COPD is unclear.

## Comparison with other studies

***Antioxidants in relation to the acute respiratory effects of air pollution***

As described in the main findings, we found a significant decrease in $FEV_1$ (186 ml) and FVC (183 ml) in the control group with an increase of 100 $\mu g/m^3$ ozone. The supplementation group (vitamin C, E and β-carotene) did, however, not show a significant effect of ozone on $FEV_1$ (-20 ml) and FVC (25 ml) (chapter 2). In chapter 3, $FEV_1$ (77 ml) and FVC (136 ml) decreased significantly with an increase of 100 $\mu g/m^3$ ozone in the control group. In the supplementation group (vitamin C and E), FVC (40 ml) decreased also significantly with an increase of 100 $\mu g/m^3$ ozone but $FEV_1$ did not decrease significantly (9 ml). The differences in ozone effect for $FEV_1$ and FVC between the control and supplementation group were statistically significant in both intervention studies.

Our results are consistent with the results of the other studies on modulation of the acute effects of ozone on pulmonary function by antioxidant supplements [10,31,33], although the level of ozone exposure and dose and sort of antioxidant supplementation were different. In one of the earlier experimental studies, a supplementation of vitamin C and E showed a mean decrease of $FEV_1$ (57 ml) and of FVC (40 ml) after two hours of exposure to 600 $\mu g/m^3$ ozone with exercise, while the placebo group showed a significantly larger mean decrement of $FEV_1$ (79 ml) and FVC (64 ml) [10]. A similar decrease in $FEV_1$ (77 ml) and in FVC (74 ml) was observed after exposure for 2 hours to 800 $\mu g/m^3$ ozone with intermittent exercise in the placebo group while the supplementation group (vitamin C, E and vegetable cocktail) showed a significantly lower decrease in $FEV_1$ (42 ml) and FVC (35 ml). These results were preliminary because of the total of 30 healthy subjects only 18 subjects were included in the analysis [33]. Both studies were experimental in design. There has only been one study under ambient conditions and that was among street workers (n=49) of Mexico-city [31]. These street workers received a similar cocktail as our cyclists in 1994 and performed repeated lung function measurements. They were, however, not exercising heavily but the ambient ozone concentration was higher (163 $\mu g/m^3$). This study had a cross-over design and in the first phase, an increase of 100 $\mu g/m^3$ ozone would significantly decrease $FEV_1$ (211 ml) and FVC (160

ml) in the placebo group while the supplement group did not decrease $FEV_1$ (46 ml) and FVC (31 ml) significantly. The second phase of this study did not show clear effects of ozone because the washout period (2 weeks) between the supplementation of antioxidants and placebos was probably too short [31].

So all studies and, in particular, the Mexican study showed similar decreases in lung function after ozone exposure in the control group and supplementation group compared to our intervention studies. The difference in decreases in lung function between the study groups were, however, smaller in the experimental studies compared to studies under ambient conditions. These experimental studies had, however, much higher levels of ozone exposure which makes comparison to the effect of ambient air pollution difficult.

To our knowledge, our panel study on a possible modulation of dietary and plasma antioxidants on the acute effects of winter air pollution was the first study investigating a possible modulation of these antioxidants in diet and in blood. So we can not compare our results quantitatively with other studies. Our results do suggest that plasma β-carotene can possibly modulate the acute effects of winter air pollutants, in particular, PM10 and black smoke on large PEF decrements.

***Antioxidants in relation to indicators of asthma and COPD***

In chapter 5, our results suggest that a high intake (213 mg) of vitamin C was associated with a 53 ml higher $FEV_1$ and a 79 ml higher FVC compared to a low intake of vitamin C (68 mg). This was consistent with other studies on vitamin C intake in relation to lung function [13,15,40]. A difference of 40 mg of vitamin C intake was associated with 25 ml higher $FEV_1$ and 23 ml higher FVC in a large (n=2,633) general population sample [13]. In the NHANES I (n=2,526) a 40 ml difference in $FEV_1$ was shown between the highest tertile (mean 178 mg) and lowest tertile (mean 17 mg) of vitamin C intake [40]. This difference (161 mg) between the highest and lowest tertile was similar compared to the difference between our $90^{th}$ and $10^{th}$ percentile of vitamin C intake (145 mg). A difference of 140 mg of vitamin C intake was associated with a difference in $FEV_1$ of 98 ml and FVC of 140 ml in a small group (n=178) of elderly subjects [15].

Subjects with a high intake of β-carotene (3.7 mg) had a 60 ml higher $FEV_1$ and a 75 ml higher FVC compared to subjects with a low intake (1.2 mg) (chapter 5). We can not compare our results quantitatively with other

studies because other published studies did not address the relation between dietary β-carotene and lung function.

Dietary vitamin E was not associated with lung function (chapter 5). This is consistent with a large population sample (n=2,633) after adjustment for vitamin C [13], but inconsistent with a small sample (n=178) of elderly [15].

In this thesis, most of the dietary antioxidants (vitamin C, E and β-carotene) were not consistently associated with the prevalence of several respiratory symptoms. This is difficult to compare quantitatively with other studies because the outcome variable, such as respiratory symptoms or disease status and the studied lag time differ between the studies. However, most prospective and cross-sectional studies did not find a relation with vitamin C and (indicators of) asthma and COPD [16,17,39]. Most studies on dietary vitamin E and β-carotene did find positive associations with respiratory symptoms or disease status as outcome [17,19].

In chapter 6, we found that cases (n=491) tended to have lower levels of plasma β-carotene than controls (n=496) (OR=0.81; 95% CI: 0.63-1.04). Both cases and controls were non-smoking adults. These lower levels of β-carotene was also observed in a group of male smokers with COPD symptoms in a cross-sectional study (n=29,133) [19]. In this study plasma β-carotene was lower in subjects with chronic bronchitis symptoms (OR=0.59; 95%CI: 0.55-0.62) and with dyspnea (OR=0.62; 95%CI: 0.58-0.66) compared to subjects without respiratory symptoms [19]. In our study, plasma α-tocopherol was not associated with respiratory symptoms but positively associated with dyspnea. However in the above-mentioned study among male smokers, plasma α-tocopherol concentration was negatively associated with chronic bronchitis and dyspnea symptoms [19].

We found that subjects with a high plasma β-carotene concentration (0.57 μmol/l) had a 147 ml higher FVC than subjects with a low plasma β-carotene concentration (0.11 μmol/l). A higher $FEV_1$ (73 ml) for high versus low levels of plasma β-carotene was observed but this difference was not statistically significant (chapter 7). The magnitude of the association was similar for FVC but not for $FEV_1$ in a cross-sectional study among 798 men who had been exposed to asbestos [43]. A difference in the concentration of serum β-carotene of 0.29 μmol/l in this study was associated with a 90 ml higher $FEV_1$ and an 82 ml higher FVC.

We did not find an association between plasma α-tocopherol and lung function. This was consistent with the only other study investigating serum α-tocopherol levels in relation to the development of airway obstruction [41].

## Biological plausibility of the associations

In the following paragraphs the biological plausibility of the associations between antioxidants and the acute respiratory effects of air pollution, and indicators of asthma and COPD is discussed.

### *Mechanisms of acute effects of air pollution*

Ozone and to a smaller extent $NO_2$ react rapidly with poly-unsaturated fatty acids (PUFAs) in the lung initiating lipid peroxidation [122]. This causes a reduction in the membrane fluidity and permeability and starts the 'oxidative stress' [123] (see mechanisms of chronic inflammation). In addition, ozone and $NO_2$ can directly release arachidonic acid from the membrane bilayer. Arachidonic acid, a precursor of prostaglandins and leukotrienes, is a poly-unsaturated fatty acid which is, in particular, prone to oxidation because of its four double bonds [123]. In this way, elevated levels of leukotrienes may cause tissue damage and attract inflammatory cells [25]. An increase in inflammatory mediators was shown in bronchoalveolar lavage within one hour [73,74], six hours [66], 18 hours [73,74], and 24 hours [66] of acute ozone exposure. This increase of inflammatory mediators was different for each mediator in time [66,73,74].

Antioxidants, in particular, vitamin C, may modulate the reactivity of oxidants (ozone and $NO_2$) by reacting directly with them before oxidants can injure the lung tissue which could have lead to increased levels of the inflammatory cells [56,122]. Vitamin E is in particular known for stopping the process of lipid peroxidation [122]. After exposure to other air pollutants, such as particulate matter and black smoke, the amount of inflammatory cells and indirect the level of oxygen metabolites can increase [124]. Antioxidants might also react with these oxygen metabolites preventing a further influx of inflammatory cells and decreasing so the damage to the lung tissues.

Since lung function decrements after ozone exposure occur earlier in time, within one hour after the exposure compared to the increase of inflammatory cells, another hypothesis was suggested: maximal inspiratory capacity was reduced through stimulation of the neural receptors in the upper airways by cyclooxygenase products of arachidonic acid [67]. This is confirmed

in another more recent study of Hazucha et al [95] who showed that an anti-inflammatory drug with antioxidant capacity (ibuprofen) suppressed the release of metabolites of arachidonic acid and decrements in lung function but not the release of inflammatory cells. Vitamins C and E have been shown to affect arachidonic acid metabolism, but the role of these vitamins in this metabolism is not fully understood [10,20,68].

***Mechanisms of asthma and COPD through chronic inflammation***

Most of the acute respiratory effects of air pollution, such as pulmonary function decrements are mild and reversible in nature, in particular, in healthy individuals. However, there are suggestions that repeated peak exposures to ambient air pollution might lead to the occurrence of asthma or COPD or deteriorate the health in those subjects in whom chronic respiratory disease is present [125-127].

Acute and chronic tissue damages of the airways are partly caused by 'oxidative stress' [128]. The term 'oxidative stress' indicates an increased free-radical formation in the body when antioxidant defences are not adequate and increased damage or cell injury is likely [129,130]. Exogenous oxidants or free radicals such as cigarette smoke and air pollution can cause direct or indirect 'oxidative stress' in the lungs [56]. These exogenous oxidants can trigger inflammatory cells, such as neutrophils, macrophages, and eosinophils to produce reactive oxygen metabolites (that is, endogenous oxidant sources) increasing further the 'oxidative stress' [25,128,131]. These inflammatory cells can also be triggered by the so-called 'respiratory burst' generating a large amount of reactive oxygen species [25]. Triggers of the 'respiratory burst' include leukotrienes, fatty acids and immune complexes [123]. During the 'respiratory burst' the inflammatory cells have an increased oxygen uptake and produce by different reactions reactive oxygen species. The produced reactive oxygen species (radicals and non-radicals) from the 'respiratory burst' can cause epithelial damage and mucus hypersecretion, important features of asthma and chronic bronchitis.

Asthma can, therefore, be considered as a chronic inflammatory process. The observed asthmatic hyperresponsiveness has been suggested to be a consequence of the inflammatory reaction and epithelial damage [25,26,131].

Chronic bronchitis has similar features as asthma, such as chronic airflow obstruction [132] and inflammation as was observed by increased

inflammatory mediators [133]. Smoking is a major cause of chronic bronchitis but not all smokers develop symptoms of chronic bronchitis [132].

Chronic increased levels of the leukocyte elastase released from neutrophils might cause emphysema. The enzyme $\alpha_1$-antitrypsin (or alpha-1-protease inhibitor) can inhibit the activity of elastase. This enzyme can be deficient by inheritance in some subjects. Cigarette smoking but also the increase of inflammatory cells such as the influx of leukocytes might increase the changes of lung tissue destruction as a result of the concomitant release of oxidative proteins which reduces $\alpha_1$-antitrypsin and thereby induce lung emphysema [24,25,56,134-136].

Antioxidants could neutralise the induced oxidative damage to lung proteins, the produced reactive oxygen species and protect against lipid peroxidation. In this way, an increased antioxidant status might decrease chronic inflammation [137]. In case of inflammation the antioxidants might additionally preserve the inhibitory activity of $\alpha_1$-antitrypsin and so the integrity of the alveolar membrane [136]. The results of a recent cross-sectional study in non-smoking subjects (n=132) showed that elevated levels of lipid peroxidation products as a measure of 'oxidative stress' and lower levels of serum bilirubine, which is an antioxidant, were associated with a lower percent predicted $FEV_1$ [138].

## Are antioxidants beneficial in asthma and COPD?

In the second part of this thesis we found that dietary vitamin C and β-carotene were associated with a higher lung function (chapter 5). Plasma β-carotene tended to be associated with a lower prevalence of respiratory symptoms (chapter 6) and with a higher lung function (chapter 7). Dietary vitamin E and plasma α-tocopherol were not associated with respiratory symptoms or lung function.

Our results should be placed in a broader perspective. Two recent β-carotene supplementation trials observed higher lung cancer incidences with daily supplementation of 20-30 mg β-carotene [44,45]. This was in particular the case in subgroups of subjects, who smoked and drank more [139]. However, baseline dietary intake and serum levels of β-carotene were inversely related to lung cancer incidence in the control groups of both trials and in other observational studies. Albanes and co-workers [139] suggested that baseline β-carotene might serve as a marker for other substances that are the actual

protective agents or that β-carotene is a general indicator for other beneficial dietary or protective life-style practices. For example, plasma levels of carotenoids showed a fairly high (r=0.54) correlation with the total intake of vegetables and fruits [140]. Additionally, an increased intake of fruits and vegetables (from 3 or less to 8 servings) for 8 weeks showed an increased plasma concentration of β-carotene, α-carotene and vitamin C [141].

Subjects with a high exposure to oxidants, such as smokers, are more likely to benefit from high intake or plasma levels of antioxidants. However, we were not able to perform statistical evaluation of the presence of effect modification of smoking status on the associations among dietary and blood levels of antioxidants, lung function and respiratory symptoms because of the relatively small number in each group for the different smoking groups. In addition, the relation between plasma antioxidants and respiratory symptoms was only studied in non-smokers. So, it remains intriguing if particular sensitive subgroups in the population, such as smokers are likely to have more benefit of the antioxidants.

In addition, we could not evaluate the independent effect of dietary vitamin C and β-carotene (chapter 5) because both antioxidants are highly correlated through their presence in the same food groups (fruits and vegetables). So we can not exclude the possibility that our results for vitamin C and β-carotene represent a healthy dietary pattern or life style rather than the effect of the antioxidants alone.

## Future studies

Since very few studies were performed to evaluate a possible beneficial effect of antioxidants on both the acute respiratory effects of ambient air pollution and on the indicators of asthma and COPD, more studies are needed. It is important to elucidate whether vitamin C, β-carotene or other antioxidants are beneficial. Furthermore, to understand if antioxidants are really the actors, which are beneficial, studies are needed to determine which other factors that could be beneficial, co-vary with antioxidants.

In order to increase our understanding intervention and observational studies on the acute effects of air pollution are helpful. Firstly, more panel/intervention studies on the acute effects of air pollution under ambient conditions should be performed rather than under laboratory conditions because a combination of pollutants which occurs under ambient conditions might be more relevant. So far most of the studies investigated a possible

modulation of the acute effects of air pollution on lung function by antioxidants. However, a modulation of the effect of air pollution on the inflammatory markers might be more important because these respiratory effects might not be as reversible as lung function decrements. Secondly, most studies on the modulation of acute effects of air pollution by antioxidants have been performed in healthy subjects while most effect might be expected from sensitive subgroups, such as subjects with asthma and COPD as was observed in chapter 4 and in a recent study under laboratory conditions [32]. Thirdly, the doses of supplements should be within the range which might be reached by a change of diet because long-term supplementation with relatively high doses (pharmacological doses) has shown to have adverse effects in studies with other outcomes, such as lung cancer [44,45]. In addition, researchers commented on these studies that synthetic supplements of β-carotene do not have the same isomer structure as naturally occurs in fruits and vegetables [142,143]. Therefore, an intervention study in which the diet is changed towards a higher intake of antioxidants with more fruits and vegetables or is supplemented with cocktails of naturally occurring antioxidants might be advisable.

For a clearer picture on the evidence of a possible beneficial effect of individual antioxidants on asthma and COPD, different studies are needed. Blood should be collected in large prospective studies as an objective marker for antioxidant status. In these studies, lung function and respiratory symptoms should be measured every few years as outcomes to determine the relevant lag time for a beneficial effect of these antioxidants. When the relevant lag time has been established, intervention studies could explain if single antioxidants, a cocktail of antioxidants or a change in diet towards more fruits and vegetables is important in preventing the occurrence of asthma and COPD. In addition, the large prospective studies could elucidate if antioxidants are only beneficial in specific subgroups, such as smokers who are exposed to high levels of oxidants.

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# SUMMARY

There is a growing recognition that air pollution even at low levels could have adverse health effects. In addition, there is an increasing recognition that the incidence of asthma has been increasing and that COPD is one of the important chronic diseases in Western countries. So new protective factors, such as antioxidants, have been evaluated in relation to the acute effects of air pollution and in relation to chronic conditions, such as asthma and COPD.

Chapter 1 describes the results of other studies on the two main research questions in this thesis. This thesis is divided into two parts and for each part one main research question was specified. In the first part (chapter 3-6), the question was whether the acute respiratory effects of air pollution under ambient conditions can be modulated by antioxidants. In the second part (chapter 5-7), we studied the associations between antioxidant (pro)-vitamins in diet and blood and the prevalence of the indicators of asthma and COPD in different population-based samples of the cross-sectional MORGEN-study (the monitoring project on risk factors and health in the Netherlands).

In chapter 2, we evaluated whether acute lung function effects of ozone can be modulated by antioxidant vitamin supplementation in 26 amateur cyclists in the summer of 1994. Repeated lung function measurements were performed before and after training sessions or competitive races on 4 to 14 occasions. The cyclists were randomly assigned to two study groups. The supplementation group (n=12) received daily antioxidant supplements (15 mg β-carotene, 75 mg vitamin E and 650 mg vitamin C) for 3 months. The control group did not receive supplementation. For each subject, post-exercise lung function was regressed on the previous 8-hour mean ozone concentration (mean 101 $\mu g/m^3$). The individual regression coefficients were pooled for each study group and weighted with the inverse of the variance. For the supplementation group, there was no effect of ozone on forced vital capacity (FVC), forced expiratory volume in 1 second ($FEV_1$), peak expiratory flow (PEF) and maximal mid-expiratory flow (MMEF). For the control group there was a significant effect of ozone on $FEV_1$, FVC and PEF. The difference between the groups was 2.08 [95% confidence interval (CI): 1.31, 2.85] $ml/\mu g/m^3$ for FVC, 1.66 (95% CI: 0.62, 2.70) $ml/\mu g/m^3$ for $FEV_1$, 6.83 (95% CI: 3.17,10.49) $ml/sec/\mu g/m^3$ for PEF and 0.42 (95% CI: -1.38, 2.22) $ml/sec/\mu g/m^3$ for MMEF.

In chapter 3, we investigated whether acute effects of ozone on lung function can be modulated by antioxidant vitamin supplementation in a placebo-controlled study. Lung function was measured in Dutch cyclists (n=38) before and after each training on a number of occasions (n=380) in the summer of 1996. The vitamin group (n=20) received daily 100 mg vitamin E and 500 mg vitamin C for 15 weeks. Individual linear regression analysis was performed with post-exercise lung function as dependent variable and 8-hour mean ozone concentration as independent variable (mean 86 $\mu g/m^3$). Medians were calculated for each study group. After excluding subjects with insufficient compliance from the analysis, a difference in ozone exposure of 100 $\mu g/m^3$ would decrease $FEV_1$ with 95 ml (95% confidence interval (95% CI): -265 to –53 ml) in the placebo group and with 1 ml (95%CI –94 to 132 ml) in the vitamin group; for FVC this was -125 ml (95% CI –384 to –36) in the placebo group and -42 ml (95% CI –130 to 35 ml) in the vitamin group. The differences in ozone effect on lung function between the control and vitamin group were statistically significant.

In chapter 4, we investigated whether a high dietary intake or serum concentration of antioxidant (pro-) vitamins could attenuate the acute respiratory effects of air pollution in panels of subjects (n=227) with chronic respiratory symptoms in the winters of 1993/1994 and 1994/1995. Subjects performed daily PEF measurements in the morning and evening and reported the occurrence of respiratory symptoms in two panels (urban and non-urban) each winter for 3 months. Logistic regression analysis was used with the prevalences of large PEF decrements (more than 10% or more than 20% below the median) or respiratory symptoms as dependent variables. The air pollutants, particles <10 $\mu m$ in diameter (PM10), black smoke (BS), $SO_2$ and $NO_2$ were the independent variables. Analyses were performed separately for subjects below and above the median levels of antioxidants (dietary vitamin C, β-carotene and serum β-carotene). In subjects with low levels of serum β-carotene (<0.30 $\mu mol/l$), air pollution, in particular, PM10 and BS was found to increase the prevalence of large PEF decrements but there was no effect on respiratory symptoms. In subjects with high levels of serum β-carotene, there were no respiratory effects of air pollution. The effect of air pollution on the prevalence of large PEF decrements and respiratory symptoms was not different for subjects with a high versus low dietary intake of vitamin C or β-carotene.

In conclusion, the results of the intervention studies described in chapter 2 and 3 suggested that antioxidant (pro)-vitamin supplementation might protect partly against acute effects of ozone on lung function in heavily exercising cyclists. The results of chapter 4 suggest that high serum levels of β-carotene attenuated the respiratory effects of air pollution, in particular, the prevalence of large PEF decrements in a panel of subjects with chronic respiratory symptoms. Dietary vitamin C and β-carotene did not modify the acute respiratory effects of winter air pollution.

In chapter 5, we examined the relations between the intake of the antioxidants (pro)-vitamins C, E and β-carotene and the presence of respiratory symptoms and lung function. Complete data were collected in the MORGEN-study in a random sample of 6,555 Dutch adults aged 20-59 years during 1994 and 1995. Antioxidant intake was assessed by a semi-quantitative food frequency questionnaire. Respiratory symptoms (cough, phlegm, productive cough, wheeze, and shortness of breath) were assessed by a self-administered questionnaire. Prevalence odds ratios for symptoms were calculated using logistic regression analysis. Linear regression analysis was used for $FEV_1$ and FVC. The results are presented as a comparison between the 90$^{th}$ and the 10$^{th}$ percentiles of antioxidant intake. Vitamin C intake was not associated with most symptoms but was inversely related with cough. Subjects with a high intake of vitamin C had a 53 ml (95%CI: 23-83 ml) higher $FEV_1$ and 79 ml (95%CI: 42-116 ml) higher FVC than those with a low vitamin C intake. Vitamin E intake showed no association with most symptoms and lung function, but had a positive association with productive cough. The intake of β-carotene was not associated with most symptoms but had a positive association with wheeze. However, subjects with a high intake of β-carotene had a 60 ml (95%CI: 31-89 ml) higher $FEV_1$ and 75 ml (95%CI: 40-110 ml) higher FVC than those with a low intake of β-carotene.

In chapter 6, we examined the relations between plasma levels of antioxidants, β-carotene and α-tocopherol, and chronic respiratory symptoms in a case-control sample with Dutch adults who were never or long-term former smokers. This sample was selected from the population-based MORGEN-study and plasma concentrations of antioxidants were determined in 496 controls and 491 cases. Cases were subjects who reported one or more respiratory symptoms. Odds ratios (ORs) were calculated for the chronic respiratory symptoms using logistic regression analysis and are presented for a difference between the 90$^{th}$ percentile (high) and the 10$^{th}$ percentile (low) of plasma antioxidant concentration. ORs were adjusted for age, gender and body mass index. The adjusted OR of plasma β-carotene for respiratory symptoms was below one (OR=0.81; 95%CI: 0.63-1.04). The OR of plasma α-tocopherol was slightly elevated for respiratory symptoms but this was mostly because of the elevated OR for dyspnea (OR=2.10; 95%CI: 1.28-3.43). Dyspnea is, however, also a common symptom in cardiovascular diseases. Additional adjustment for cardiovascular risk factors did not change the positive association between dyspnea and plasma α-tocopherol.

In chapter 7, we investigated the relation between plasma antioxidants (β-carotene and α-tocopherol) and lung function in Dutch adults. A random sample (n=367) was drawn from the participants in the MORGEN study in 1995. Linear

regression analysis was performed with $FEV_1$ or FVC as dependent variables and plasma antioxidant concentration as independent variable. We adjusted for age, height, gender, smoking status, pack-years of smoking, and alcohol consumption and we presented the results as a difference between the 90th and 10th percentile of plasma antioxidant concentration. Subjects with a high plasma β-carotene level tended to have a higher $FEV_1$ (73 ml, standard error of the mean (SEM) 60 ml; p=0.22) and a higher FVC (147 ml, SEM 76 ml; p=0.052) than subjects with a low plasma β-carotene level. This difference in lung function was only observed in current and never smokers but not in former smokers. There was no difference in lung function between subjects with high and low plasma α-tocopherol concentrations.

In conclusion, we found that dietary vitamin C and β-carotene were positively associated with $FEV_1$ and FVC (chapter 5). Plasma β-carotene tended to be positively associated with $FEV_1$ and FVC but this was not statistically significant for $FEV_1$ (chapter 7). Dietary vitamin E (chapter 5) and plasma α-tocopherol (chapter 7) were not associated with lung function. Plasma β-carotene tended to be inversely associated with respiratory symptoms. Plasma α-tocopherol was not associated with asthma or COPD symptoms but was positively associated with dyspnea (chapter 6). None of the dietary antioxidants (vitamin C, E and β-carotene) were consistently associated with respiratory symptoms (chapter 5).

In chapter 8, we conclude that measurement error in dietary antioxidants measured with our food frequency questionnaire is relatively large and that blood levels of antioxidants might better reflect the concentration of the antioxidants in the lungs. Our results of the intervention studies are comparable with those of other studies, both under ambient and laboratory conditions. Our results of the beneficial association of dietary vitamin C and plasma β-carotene on lung function were also observed by other cross-sectional studies. The lack of an association of dietary vitamin E and plasma α-tocopherol on lung function and respiratory symptoms is inconsistent with other studies. Future studies are needed to elucidate the mechanism of the observed associations. The following questions are important to take into account: (1) could antioxidants attenuate the effect of air pollution on inflammatory markers, (2) what is the relevant lag time for a beneficial effect of antioxidants, in particular, vitamin C and β-carotene on indicators of asthma and COPD and (3) are antioxidants vitamin C and/or β-carotene or other substances associated with these antioxidants or a healthy life style the actual beneficial factors in relation to indicators of asthma and COPD.

# SAMENVATTING

*Met deze Nederlandse samenvatting geef ik kort de inhoud van mijn proefschrift weer. Ik heb hem geschreven voor de belangstellenden die niet bekend zijn met het vakgebied epidemiologie en het onderwerp, zoals familie en vrienden.*

Er bestaat een toenemend besef dat lage concentraties luchtverontreiniging zelfs slecht zijn voor de gezondheid, vooral voor de luchtwegen en longen. Ook is duidelijk geworden dat het aantal mensen dat astma krijgt toeneemt en dat chronische obstructieve longziekten (COPD) een belangrijke chronische ziekten categorie vormen in Westerse landen. Daarom wordt er gezocht naar nieuwe mogelijk beschermende factoren in de voeding, zoals antioxidanten, in relatie tot de acute effecten van luchtverontreiniging en in relatie tot chronische ziekten, zoals astma en COPD. Het begrip COPD omvat de ziekten chronische bronchitis en longemfyseem.

In hoofdstuk 1 beschrijf ik de resultaten van gepubliceerd wetenschappelijk onderzoek op het gebied van de twee vraagstellingen die centraal staan in dit proefschrift. Dit proefschrift is opgesplitst in twee delen. In het eerste deel (hoofdstuk 2, 3, 4) staat de vraag centraal of acute effecten van luchtverontreiniging in de buitenlucht op de luchtwegen kunnen worden tegengegaan door antioxidanten. In het tweede deel (hoofdstuk 4, 5, 6) staat de vraag centraal of er een verband is tussen antioxidant vitamines en de aanwezigheid van astma en COPD. De onderzochte antioxidant vitamines in dit proefschrift zijn vitamine C, E en β-caroteen. Vitamine C en β-caroteen komen voornamelijk voor in groente en fruit en vitamine E in noten en plantaardige oliën en vetten.

In hoofdstuk 2 is gekeken of de acute effecten van ozon op longfunctie veranderd kunnen worden door het geven van voedingssupplementen met antioxidant vitamines (capsules) bij 26 amateur wielrenners gedurende de zomer van 1994. Per persoon werden er 4 tot 14 keer longfunctiemetingen uitgevoerd voor en na de trainingen of wedstrijden. Aan het begin van het onderzoek werden de wielrenners willekeurig toegewezen aan één van de twee groepen. In de antioxidanten groep zaten 12 wielrenners die dagelijks antioxidant capsules gedurende 3 maanden kregen (15 mg β-caroteen, 75 mg vitamine E, en 650 mg vitamine C). De controle groep bestond uit 14 wielrenners die geen capsules kregen.

De gemiddelde ozon concentratie over de 8 uur voorafgaand aan de longfunctiemetingen na trainingen/ wedstrijden was 101 $\mu g/m^3$ gedurende de onderzoeksperiode. Na statistische analyse bleek dat er in de antioxidanten groep geen effect was van ozon op de volgende longfunctieparameters: $FEV_1$ (=volume lucht die in 1 seconde kan worden uitgeblazen bij een geforceerde uitademing), FVC (=totale longinhoud bij een geforceerde uitademing), PEF (ook wel piekstroom genoemd en is gelijk aan de maximale uitademingssnelheid). De controle groep liet wel een statistisch significant effect van ozon op de $FEV_1$, FVC en PEF zien, namelijk de longfunctie daalde bij hogere ozon concentraties in de buitenlucht. Het verschil tussen de controle en antioxidanten groep in het effect van ozon was significant voor de meeste longfunctie variabelen ($FEV_1$, FVC en PEF).

Hoofdstuk 3 gaat over een onderzoek uitgevoerd in 1996 die vergelijkbaar is met het onderzoek in 1994. Alleen werden er meer personen gemeten en de controle groep kreeg placebo capsules (neppillen). Longfunctie werd in totaal 380 keer gemeten bij 38 wielrenners voor en na de trainingen gedurende de zomer van 1996. De wielrenners werden willekeurig toegewezen aan een van de beide groepen. De vitamine groep kreeg dagelijks 100 mg vitamine E en 500 mg vitamine C voor 15 weken. De controle groep kreeg dagelijks placebo capsules die zichtbaar niet waren te onderscheiden van de vitamine capsules. De gemiddelde ozon concentratie over de 8 uur voorafgaand aan de longfunctiemetingen na trainingen was 86 $\mu g/m^3$ gedurende deze onderzoeksperiode. Na het uitsluiten van personen in de statistische analyses die niet genoeg capsules hadden geslikt, was er een significant effect van ozon op de longfunctie in de controle groep, maar niet in de vitamine groep. Uitgedrukt in getallen gaf een verschil in ozon blootstelling van 100 $\mu g/m^3$ een 95 ml afname in $FEV_1$ in de controle groep en een 1 ml afname in vitamine groep. Voor FVC was de afname 125 ml in de controle groep en 42 ml in de vitamine groep. De verschillen in het effect van ozon op de longfunctie tussen de controle en vitamine groep was statistisch significant.

In hoofdstuk 4 onderzochten we of een hoge inname via de voeding of een hoge bloed concentratie van antioxidant vitamines de acute effecten van luchtverontreiniging op de luchtwegen kunnen tegengaan. Het onderzoek vond plaats gedurende 2 winters (1993/1994 & 1994/1995) bij 227 volwassenen van 50 tot 70 jaar oud met chronische luchtwegklachten. De antioxidanten inname via de voeding werd gemeten met behulp van een voedselfrequentievragenlijst. Dit

is een vragenlijst waarin de frequentie van een aantal voedingsmiddelen, die veel gegeten worden door de algemene bevolking, wordt nagevraagd. De piekstroom (PEF) werd 's ochtends en 's avonds dagelijks voor 3 maanden gemeten door de deelnemers zelf en de uitkomsten worden samen met het voorkomen van luchtwegklachten opgeschreven in een dagboekje. In dit onderzoek werd onder andere gekeken naar de verbanden tussen het vóórkomen van grote dalingen in piekstroom of luchtwegklachten met luchtverontreinigingsconcentraties van fijn stof met een diameter kleiner dan 10μm (PM10), zwarte rook, zwaveldioxide ($SO_2$) en stikstofdioxide ($NO_2$). Deze verbanden werden apart bestudeerd voor mensen met hoge en lage niveaus van antioxidanten in de voeding (vitamine C, en β-caroteen) of β-caroteen in bloed. Uit de resultaten bleek dat personen met lage niveaus van β-caroteen in bloed vaak grote piekstroom dalingen hadden na verhoogde luchtverontreinigingsconcentraties. Bij personen met hoge niveaus van β-caroteen in bloed waren er geen grote piekstroom dalingen na verhoogde luchtverontreingings-concentraties. De acute effecten van luchtverontreiniging op de luchtwegen waren niet verschillend voor personen met hoge of lage inname van vitamine C of β-caroteen via de voeding.

Uit voorgaande drie hoofdstukken kunnen de volgende conclusies getrokken worden. De twee interventie onderzoeken in hoofdstuk 2 en 3 suggereren dat antioxidant vitamine supplementen mogelijk zouden kunnen beschermen tegen de effecten van ozon op de longfunctie bij zich hevig inspannende wielrenners. De resultaten van hoofdstuk 4 suggereren dat hoge niveaus van β-caroteen in bloed de acute effecten van luchtverontreiniging in de winter kunnen tegen gaan bij een groep volwassenen met chronische luchtwegklachten. Vitamine C en β-caroteen inname via de voeding veranderde de acute effecten van luchtverontreiniging in de winter niet.

In het tweede deel van dit proefschrift (hoofdstuk 5,6,7) werd het verband tussen niveau's van antioxidant vitamine inname via de voeding en in bloed en de aanwezigheid van astma en COPD onderzocht in verschillende steekproeven van de bevolking. De steekproeven kwamen voort uit een dwarsdoorsnede onderzoek naar risicofactoren en de aanwezigheid van chronische ziekten in de Nederlandse bevolking bij de leeftijdsgroep van 20 tot 59 jaar. Dit onderzoek is uitgevoerd op de GGD-en in 3 steden, namelijk Amsterdam, Doetinchem en Maastricht. Dit onderzoek heet het MORGEN-project en de afkorting staat voor het MOnitoring project over Risicofactoren en GEzondheid in Nederland.

In hoofdstuk 5 bestudeerden we het verband tussen de inname van antioxidant vitamines via de voeding en de aanwezigheid van luchtwegklachten en longfunctie. Complete gegevens van 6.555 volwassenen waren verzameld in het MORGEN-project gedurende 1994 en 1995. Antioxidant inname (vitamine C, E en β-caroteen) werd gemeten met behulp van een voedselfrequentie vragenlijst. Chronische luchtweg-klachten in het afgelopen jaar (hoesten, slijm opgeven, hoesten met slijm opgeven, piepen op de borst, kortademigheid) werden nagevraagd met een schriftelijke vragenlijst. Na statistische analyse bleek dat vitamine C inname niet gerelateerd was aan de meeste luchtwegklachten. Personen met een hoge inname hadden, echter, wel minder hoestklachten. Personen met een hoge inname van vitamine C hadden een significant hogere $FEV_1$ (53 ml) en FVC (79 ml) dan personen met een lage inname vitamine C. Vitamine E vertoonde geen verband met de meeste luchtwegklachten en met longfunctie. Personen met een hoge inname van vitamine E hadden, echter, wel meer klachten van hoesten met slijm opgeven. De inname van β-caroteen hing niet samen met de meeste luchtwegklachten, maar personen met een hoge inname hadden meer klachten van piepen op de borst. Echter, personen met een hoge inname van β-caroteen hadden een significant hogere $FEV_1$ (60 ml) en FVC (75 ml) dan personen met een lage inname van β-caroteen.

In hoofdstuk 6 beschrijven we een onderzoek naar het verband tussen niveaus van antioxidanten in bloed (β-caroteen en α-tocopherol) en chronische luchtwegklachten in een patiënt-controle steekproef bij volwassenen die nooit of langer dan 10 jaar geen sigaretten hebben gerookt. Deze steekproef kwam uit het MORGEN-project en de antioxidantconcentraties in bloed zijn bepaald bij 496 controles (mensen zonder luchtwegklachten) en 491 patiënten (mensen met één of meer chronische luchtwegklachten). Het verband tussen antioxidanten en chronische luchtwegklachten werd gecorrigeerd voor leeftijd, geslacht en Quetelet Index (maat voor overgewicht, uitgedrukt als het gewicht in kg / lengte in meters$^2$). Na correctie voor deze factoren hadden personen met luchtwegklachten een lager niveau van β-caroteen in bloed dan personen zonder luchtwegklachten. De concentratie van α-tocoferol in het bloed was niet verschillend tussen personen met en zonder luchtwegklachten. Alleen personen met kortademigheidklachten hadden een hogere concentratie α-tocoferol in bloed dan personen zonder chronische luchtwegklachten. Kortademigheid is,

echter, ook een belangrijk symptoom voor personen met hart- en vaatziekten. Na correctie voor risico factoren van hart- en vaatziekten hadden mensen met kortademigheidklachten nog steeds hogere α-tocoferol niveaus in hun bloed.

In hoofdstuk 7 onderzochten we het verband tussen antioxidanten (β-caroteen en α-tocoferol) in bloed en longfunctie in een groep volwassenen. Een steekproef werd getrokken van bijna 400 mensen uit alle deelnemers van het MORGEN project waarvan gegevens waren verzameld in 1995. Het verband werd voor de volgende variabelen gecorrigeerd: leeftijd, lengte, geslacht, rookstatus (nooit roker, ex-roker of huidig roker), het aantal pakjes-jaren van roken (bestaat uit de duur van roken in jaren en het gemiddeld aantal pakjes sigaretten wat men gerookt heeft) en alcohol consumptie. Uit de resultaten bleek dat na correctie voor deze variabelen, personen met een hoge concentratie van β-caroteen in bloed een hogere $FEV_1$ (73 ml) en een hogere FVC hadden (147 ml) dan personen met een lager β-caroteen niveau in bloed. Het verband tussen β-caroteen in bloed en $FEV_1$ was echter niet significant. Een hoger longfunctie niveau bij een hoger bloed niveau van β-caroteen werd alleen waargenomen bij personen die nu sigaretten rookten en die nooit sigaretten hebben gerookt, maar niet bij ex-rokers van sigaretten. Er was geen verschil in het longfunctie niveau tussen personen die hoge en lage concentraties van α-tocoferol in bloed hadden.

De volgende conclusies kunnen getrokken worden uit de drie voorafgaande hoofdstukken. Een hogere inname van vitamine C en β-caroteen via de voeding en een hoger niveau van β-caroteen in bloed hingen samen met het hebben van een betere longfunctie. Vitamine E inname via de voeding en α-tocoferol niveau in bloed hingen niet samen met longfunctie. Personen met chronische luchtwegklachten hadden een lagere concentratie β-caroteen in het bloed dan personen zonder luchtwegklachten. De concentratie α-tocoferol in bloed was niet verschillend voor personen met astma- en COPD-achtige klachten, maar was hoger in personen met klachten van kortademigheid. Geen van de antioxidanten uit de voeding hing eenduidig samen met de aanwezigheid van chronische luchtwegklachten.

In hoofdstuk 8 concluderen we dat het aantal meetfouten bij het bepalen van antioxidanten in de voeding met behulp van onze voedselfrequentie vragenlijst groot is. Niveaus van antioxidanten in het bloed geven mogelijk beter de concentratie in de longen weer dan antioxidanten gemeten in de voeding. Onze resultaten van beide onderzoeken bij wielrenners dat antioxidanten een

mogelijke bescherming biedt tegen het effect van ozon op de longfunctie, zijn vergelijkbaar met de resultaten van andere onderzoeken die zowel zijn uitgevoerd onder laboratorium omstandigheden als in de buitenlucht. De gunstige resultaten van de verbanden tussen vitamine C inname via de voeding, β-caroteen niveau in bloed en longfunctie zijn vergelijkbaar met andere onderzoeken. Het gebrek aan effect van vitamine E inname via de voeding en α-tocoferol concentratie in bloed op de longfunctie of luchtwegklachten komt niet altijd overeen met de resultaten van andere onderzoeken.

Nieuwe onderzoeken zijn nodig in de toekomst om het mechanisme dat de gevonden resultaten kan verklaren, op te helderen. Voorbeelden van vraagstellingen zijn: (1) Kunnen antioxidanten ook het effect van luchtverontreiniging op de ontstekingscellen (deze zijn verhoogd bij onder andere astmapatiënten) beïnvloeden; (2) Wat is de relevante tijdsperiode waarin antioxidanten (vitamine C en β-caroteen) de longfunctie of luchtwegklachten kunnen beïnvloeden; en (3) Zijn het wel de antioxidanten (vitamine C en β-caroteen) zelf die de gunstige werking laten zien of zijn het andere stoffen of factoren zoals een gezonde levensstijl die sterk samenhangen met de antioxidanten.

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Linda Grievink was born in Olst in the Netherlands on June 5th 1969. After secondary school (Jacob Revius Scholengemeenschap in Deventer), she started the M.Sc. program of Human Nutrition at the Department of Nutrition, Wageningen Agricultural University, Wageningen in 1988. She graduated with majors in Nutrition and Epidemiology in 1993 after a five month training period at Monash University in Melbourne, Australia. From October 1993 until February 1998, she was appointed as a PhD-fellow at the former department of Epidemiology and Public Health at the Wageningen Agricultural University for four years and as a research associate at the National Institute of Public Health and the Environment (RIVM), in Bilthoven, the Netherlands for four months. During this period, she finished the research described in this thesis. In May 1998, she started as a researcher working on the methodology of dietary patterns at the Department of Chronic Diseases and Environmental Epidemiology of the National Institute of Public Health and the Environment, Bilthoven, the Netherlands.

Linda Grievink werd op 5 juni 1969 geboren in Olst. Na het behalen van het HAVO en VWO-diploma op het Jacob Revius Scholengemeenschap te Deventer, begon zij in 1988 met de studie Voeding van de mens aan de Lanbouwuniversiteit te Wageningen. Deze studie in de richting 'voeding en gezondheid' werd in 1993 afgerond met doctoraalonderzoeken in de voeding en epidemiologie en met een stage in de epidemiologie bij de 'Monash University' in Melbourne, Australië. Van oktober 1993 tot en met februari 1998 was ze aangesteld als assistant in opleiding (AIO) op de voormalige vakgroep Humane Epidemiologie en Gezondheidsleer aan de Lanbouwuniversiteit. Tijdens deze aanstelling voerde ze de onderzoeken beschreven in dit proefschrift uit en heeft ze gedurende vier maanden een aanstelling gehad als toegevoegd onderzoeker op het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) in Bilthoven. In mei 1998 werd ze als projectmedewerker aangesteld bij het Centrum voor Chronische ziekte en Milieu epidemiologie (CCM) van het RIVM.