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# Dietary flavonoids and chronic respiratory diseases in Italian adults

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*Dietary flavonoids and chronic respiratory diseases in Italian adults.*
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# Sommario

**Introduzione e obiettivi:** I flavonoidi sono bio-composti presenti in quantità consistenti in alcuni tipi di frutta, verdura, erbe e vino rosso. Grazie al loro effetto antiossidante e antinfiammatorio sulle vie aeree, è stato ipotizzato che i flavonoidi possano ridurre la gravità e/o prevenire il rischio di malattie polmonari. Lo scopo di questa tesi è stimare l'associazione tra asma (corrente e in passato), bronchite cronica, rinite (allergica e non) e assunzione di flavonoidi con la dieta (flavonoidi totali e sottoclassi principali: flavanoni, antociani, flavan-3-oli, flavonoli, flavoni, polimeri e proantocianidine).

**Metodi:** Sono stati analizzati i dati dello studio multicaso-controllo Gene-Environment Interaction in Respiratory Diseases (GEIRD). Per lo studio sono stati selezionati soggetti fra i $20$ ed i $84$ anni di età dalla popolazione generale. Per raccogliere le informazioni sulle abitudini alimentari, è stato utilizzato il questionario EPIC (European Prospective Investigation into Cancer and Nutrition). Si sono utilizzati modelli di regressione multinomiale per valutare l'associazione tra esposizioni dietetiche e il rapporto di rischio relativo (RRR) per ogni patologia, aggiustando per età, sesso, centro, indice di massa corporea, abitudine al fumo, consumo di alcol, istruzione, apporto energetico totale, vitamina C e assunzione totale di frutta. I soggetti inclusi nelle analisi erano $990$, gerarchicamente definiti come segue: soggetti con asma (corrente, CA, $n = 159$; in passato, PA, $n = 78$), bronchite cronica (CB, $n = 47$), rinite allergica (allergica, AR, $n = 167$; non allergica, NAR, $n = 142$) e controlli ($n = 397$).

**Risultati:** Un aumento di una deviazione standard nell'assunzione di flavanoni è associato ad un rischio ridotto di NAR (RRR aggiustato $= 0.68$, intervallo di confidenza al $95\%$ (IC) $0.47; 0.97$); un risultato simile è stato trovato confrontando

il quartile più alto rispetto a quello più basso dell'assunzione di flavanoni (RRR aggiustato $= 0.24$, IC al $95\%$ $0.10; 0.59$).

**Conclusioni:** I flavanoni, principalmente contenuti negli agrumi, potrebbero ridurre il rischio di NAR. Non sono state trovate associazioni significative tra l'assunzione di flavonoidi e probabilità di sviluppare CA, PA, CB oppure AR.

# Abstract

**Background and objectives:** Flavonoids are bio-compounds widely found in fruits, vegetables, herbs and red wine. Due to their antioxidant and anti-inflammatory effect in the airways, flavonoids have been suggested to reduce the severity or prevent the risk of lung diseases. The aim of this thesis is to assess the association between asthma (current and past), chronic bronchitis and rhinitis (allergic and non-allergic) and dietary intake of flavonoids (total and the major subclasses: flavanones, anthocyanins, flavan-3-ols, flavonols, flavones, polymers and proanthocyanidins).

**Methods:** Data from Gene-Environment Interaction in Respiratory Diseases (GEIRD), a multi-case control study, was analysed. Subjects aged between $20$ and $84$ years old were selected from general population. To ascertain dietary intake, the European Prospective Investigation into Cancer and Nutrition Food Frequency Questionnaire (EPIC) was used. Multinomial regression models were used to assess the association between dietary exposures and the relative risk ratio (RRR) of being a case adjusting for age, sex, centre, body mass index, smoking habit, alcohol intake, education, total energy intake, vitamin C and total fruit intake. The subjects included in the analyses were $990$, hierarchically defined as follows: subjects with asthma (current, CA, $n = 159$; past, PA, $n = 78$), chronic bronchitis (CB, $n = 47$), allergic rhinitis (allergic, AR, $n = 167$; non-allergic, NAR, $n = 142$) and controls ($n = 397$).

**Results:** An increase of 1 standard deviation of flavanones was associated with a reduced risk of NAR (adjusted RRR=$0.68$, $95\%$ Confidence Interval (CI) $0.47; 0.97$); a similar result was found comparing the highest vs. lowest quartile of flavanones intake (adjusted RRR=$0.24$, $95\%$ CI $0.10; 0.59$).

**Conclusions:** Flavanones, which are mainly contained in citrus fruits, might

reduce the risk of NAR. No significant associations were found between dietary intake of flavonoids and CA, PA, CB or AR.

# Contents

# Chapter 1

# Introduction

## 1.1 Pathophysiological mechanisms and epidemiology of asthma

Asthma is a disorder characterized by chronic inflammation and airways hyperresponsiveness that cause episodes of wheezing, breathlessness, chest tightness, and coughing. A variable level of airflow obstruction, often reversible with treatment or spontaneously, is often associated with these symptoms. [1, 2] In the pathogenesis of asthma, reactive oxygen and nitrogen species, that originate from the inflammatory cells recruited in the lung tissue, play an important role. [3] Superoxide dismutase (SOD), which is an important antioxidant in cells, might be inactivated by oxidative and nitrosative stress, which in turn lead to a damage and remodeling of the lung tissue. [4] Furthermore, a large body of evidence reported that SOD activity is reduced in airway epithelial cells of subjects affected by asthma, probably due to the inflammation. [5] According to an increasing number of studies, the dietary intake of antioxidants might influence the oxidant/antioxidant balance, which is thought to be altered in asthmatic patients. [3]

Asthma is one of the main pulmonary disease in the world among subjects of any age and it has been estimated that in $2025$ asthma patients will be more than $100$ million. [1] The global prevalence in adults is between $1\%$ to $21\%$, whilst up to $20\%$ of children between $6$ and $7$ years of age experience severe wheezing events

within a year. [6, 7] Subjects affected by asthma often require the use of emergency care, sometimes followed by hospital admission and they are at risk of permanent disability and premature death. [1] Asthma costs are very high and it significantly impact social life by causing a high number of missed school and/or work days. [8, 9]

## 1.2 Pathophysiological mechanisms and epidemiology of chronic bronchitis

Chronic bronchitis, one of the clinico-pathological entities of chronic obstructive pulmonary disease (COPD), is defined by clinicians as the presence of persistent cough with sputum expectoration for at least three months a year. [10] The condition is caused mainly by the cigarette smoke or inhalation of noxious gases and fumes, which lead to chronic inflammation in the bronchial walls, with infiltration of neutrophils and macrophages. [11] Chronic bronchitis is mainly characterized by an augmented production of mucus as consequence of an increased number of goblet cells and expansion of the mucus-secreting glands. [12] The lumen in the smaller airways is reduced due to inflammation, fibrosis and the presence of an excess of mucus. [12] In a study conducted on $25$ patients, it was found that in patients affected by chronic bronchitis the augmented presence of inflammatory cells in bronchial epithelium and in submucosa are associated with a reduced forced expiratory volume in the 1st second ($FEV_1$), both in smokers and non-smokers subjects. [10]

During the 60ies chronic bronchitis, emphysema and asthma were thought to have the same pathogenesis. [13] This theory, called “Dutch hypothesis”, was later opposed by the so called “British hypothesis”, according which asthma and COPD originate from different causal mechanisms. [14, 15] Dutch hypothesis would be reinforced by longitudinal studies that found a correlation between asthma in children and later onset of fixed airflow obstruction chronic obstructive pulmonary disease (COPD), [16] and by the fact that the reversibility of obstruction is present in both the diseases in some cases. [17] However, the study published in $2006$ by Peter J.

Barnes supports the British hypothesis, arguing that the inflammation mechanisms in asthma and COPD are different because the cells involved are not the same type. [18]

Chronic bronchitis, which affects more men than women, has a prevalence that spans from $3.4$ to $22\%$ in adults, but it is more common in subjects affected by chronic COPD, with a prevalence that might reach the $74\%$. [19] According to recent investigations, subjects affected by chronic bronchitis symptoms tend to have and increased lung function decline and are at higher risk of COPD and death. [20] There is also an evidence suggesting that chronic bronchitis symptoms in subjects with normal lung function might affect the quality of life, increase the risk of lung function damage and the risk of respiratory exacerbations. [21, 22] Despite chronic bronchitis symptoms are widely reported and despite the important findings related to this issue, this condition did not receive sufficient attention from researchers and clinicians. [20]

## 1.3 Pathophysiological mechanisms and epidemiology of rhinitis

Rhinitis is a chronic condition characterized by inflammation of the nasal mucosa and the presence of one or more symptoms among sneezing, itching, nasal discharge and nasal blockage. [23] Rhinitis can have many phenotypes, but the most common non-infectious form is the allergic rhinitis, in which environmental allergens lead to an IgE-mediated immune response. [24] In non-allergic rhinitis, the symptoms are not triggered by any allergens and the subject is negative to allergen tests. [23]

In nasal airway epithelium there are ciliated cells, mucus-secreting goblet cells and basal cells that constitute a connection between the environment and the immune system. [25] The pathogenesis of rhinitis involves the mucociliary clearance mechanism, which might be impaired in inflammatory conditions, leading to an accumulation of mucus; inflammation also causes nasal congestion due to the augmented permeability of the blood vessels. [23]

In adults, the adjusted prevalence for non-allergic rhinitis is $9.6\%$ and $29.8\%$ for

the allergic phenotype, with a female predominance of the non-allergic rhinitis and male predominance of the allergic phenotype. [23] According to epidemiological evidence, subjects with allergic or non-allergic rhinitis are at high risk to develop asthma. [26] Allergic rhinitis prevalence is increasing, significantly impacting the quality of life, work productivity and medical treatment costs. [22, 27]

## 1.4 The antioxidant and anti-inflammatory properties of flavonoids

Flavonoids (Figure 1.1) are a large class of compounds that belongs to polyphenols, synthetized by plants and usually found in form of glycosides. [28] Plant origin foods, such as vegetables, fruits, chocolate, wine and tea are source of flavonoids in the diet. [29] The flavonoids content of the foods were described by the United States Department of Agriculture (USDA) and the tables were reported by Seema Bhagwat and David B. Haytowitz. [30, 31] The following 26 dietary flavonoids, categorized in 5 subclasses, are widely found in vegetable foods and were estimated by USDA for 506 items:

- FLAVONOLS: Isorhamnetin, Kaempferol, Myricetin, Quercetin
- FLAVONES: Apigenin, Luteolin
- FLAVANONES: Eriodictyol, Hesperetin, Naringenin
- FLAVAN-3-OLS: (+)-Catechin, (+)-Gallocatechin, (-)-Epicatechin, (-)-Epigallocatechin, (-)-Epicatechin 3-gallate, (-)-Epigallocatechin 3-gallate, Theaflavin, Theaflavin 3-gallate, Theaflavin 3'-gallate, Theaflavin 3,3'-digallate, Thearubigins
- ANTHOCYANIDINS: Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin

Proanthocyanidins (PAs), also called "Condensed Tannins", are oligomers and polymers of flavan-3-ols. The content of this class of flavonoids was described for 283 foods by USDA. [31]

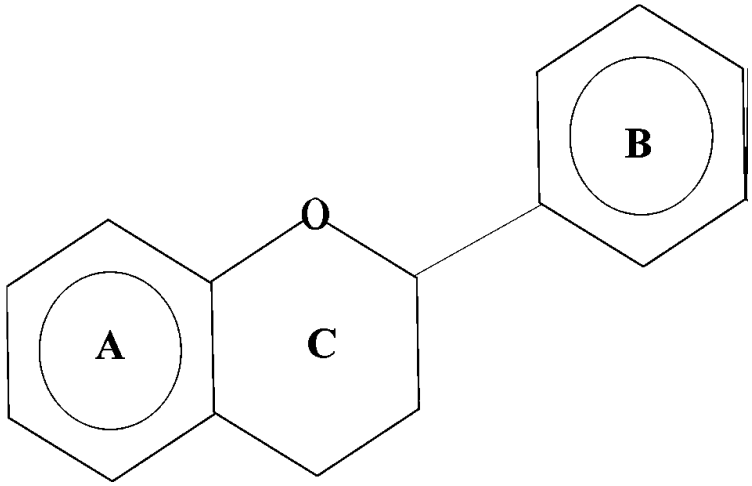
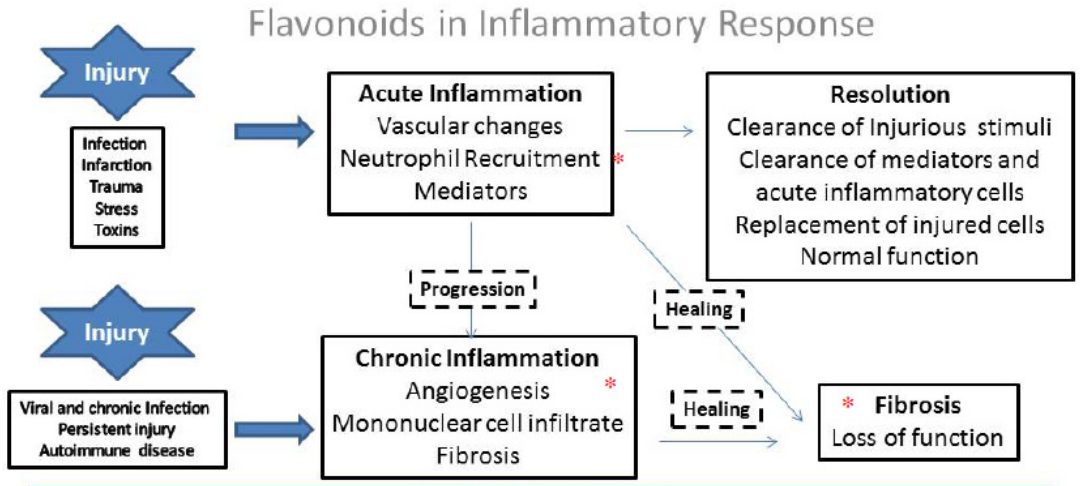



Figure 1.1: Basic flavonoid structure. (Source: Ross JA *et al.*, 2002)

According to several experimental studies, flavonoids have been shown to have antioxidant, anti-allergic and anti-inflammatory properties (Figure 1.2). [28, 29, 32–36] It has been demonstrated that the activation of Cyclooxygenase (COX) gene, which participate in the inflammatory response, can be reduced by




| Cells involved in inflammatory response | |
|---|---|
| Mast Cell | Source of mediators such as histamine and others * |
| Macrophage | Elimination of microbes and dead tissue; source of mediators such as cytokines and other; role in immune response * |
| Endothelial Cell | Source of mediators such as nitric oxide, cytokine, others * |
| Polymorphonuclear leucocytes | Elimination of microbes, dead tissue and source of inflammatory mediators such nitric oxide and others * |
| Plasma Proteins | Complement: mediators of inflammation, elimination of microbes |
| Lymphocyte | Immune response and source of cytokines |
| Monocytes | Interaction with macrophage |
| Fibroblast and ECM proteins and cells | Repair * |

Figure 1.2: Inflammatory response and cells involved in inflammatory cascade. * shows the steps that provide evidence that flavonoids can act to counteract the inflammatory response. (Source: Ross JH *et al.*, 2014)

flavonoids. [37, 38] An antioxidant mechanism of flavonoids might be the inhibition of lipoxygenase, whose products contribute to the pathological mechanisms of rhinitis and other inflammatory diseases. [39] It seems that flavonoids might neutralize the peroxyl radical complexes, which are used by lipoxygenase to synthetize arachidonic acid. [40]

While the potential beneficial effects of flavonoids have been largely demonstrated by experimental studies, epidemiological evidence until now is limited and only few flavonoids categories have been studied. [41] High intake of quercetin, naringenin, and hesperetin was associated with a reduced risk of asthma in a cohort of Finnish adults. [42] In another study conducted in London, no evidence of a beneficial effect of catechins, flavonols and flavones intake on asthma and chronic bronchitis was found. [43]

## 1.5 Aim of the thesis

Flavonoids, according to experimental studies, have antioxidant, anti-allergic and anti-inflammatory properties that might counteract the inflammation in chronic respiratory diseases. Being that epidemiological evidence on flavonoids and pulmonary health is limited ad unclear, we aimed to explore the effect that flavonoids might have on chronic respiratory diseases by analyzing data from the GEIRD study (Gene-Environment Interaction in Respiratory Diseases). According to a recent review on medicinal plants, flavonoids, thanks to their ability to intervene on the lung inflammatory pathway, are considered a promising treatment to counteract chronic respiratory diseases. [44]

We estimated flavonoids intake (total and the main subclasses: flavanones, anthocyanins, flavan-3-ols, flavonols, flavones, polymers and proanthocyanidins) using the information from a food frequency questionnaire (FFQ). According to a recent study conducted in Australia, FFQ is a helpful tool to accurately estimate the daily intake of different classes of flavonoids in middle-aged general population, which is also sensible enough to catch the differences among different countries; moreover, this is still a poorly explored field, as no many studies estimated the

flavonoids classes. [45]

# Chapter 2

# Methods

## 2.1 Study Design

GEIRD is a population-based multi-case-control study conducted in Italy that aimed to collect data on environmental exposures, history of disease, treatment, genetic information and measurements of markers of inflammation, involving subjects affected by chronic respiratory diseases (asthma, allergic rhinitis and chronic obstructive pulmonary disease (COPD)). [46] The study involved seven Italian centres and was structured in 2-stages. The aim of the first stage was to detect the potential cases and controls by using a screening questionnaire on respiratory symptoms. [46] The questionnaire was sent by e-mail to a random samples from the general population ($20 - 84$ years of age, male/female=1/1) and to pre-existing random cohorts (the Italian Study on Asthma in Young Adults (ISAYA), [47] the Italian arm of the European Community Respiratory Health Survey (ECRHS-Italy) [48]). The age range of both ISAYA and ECRHS-Italy cohort was $20 - 44$ years. The second stage consisted in a clinical visit to ascertain the respiratory condition of the participants. All the subjects who reported signs suggestive of chronic bronchitis (CB), COPD or asthma, a random sample of subjects who reported signs suggestive of rhinitis and a random sample of subjects free from any respiratory symptoms were invited to clinics. Each participant underwent a computer-assisted clinical interview, pulmonary function tests, methacholine test, reversibility test, skin prick test (SPT) while a self-administered food frequency questionnaire (FFQ) was used to collect

information on the dietary habits of the subjects. Through the clinical interview, information on lifestyle factors were collected. The level of physical activity was assessed on the following questions:

- How often do you usually exercise so much that you get out of breath or sweat?
- How many hours a week do you usually exercise so much that you get out of breath or sweat?

In relation to smoking habits, the following information were asked to the participants:

- Ever smoked for as long as a year
- Age when started smoking
- Currently smoke as of one month ago
- Number of cigarettes smoked per day
- Number of cigarillos smoked per day
- Number of cigars smoked per week
- Amount of pipe tobacco smoked in grams per week
- Stopped or cut down smoking
- Stopped or cut down due to breathing problems
- Age when stopped or cut down smoking
- Number of cigarettes smoked per day before cutting down
- Number of cigarillos smoked per day before cutting down
- Number of cigars smoked per week before cutting down
- Amount of pipe tobacco smoked in grams per week before cutting down

All measurement protocols were in agreement with international guidelines (www.geird.org). [46] Only the subjects recruited in the years from 2007 to 2010 in the centres of Pavia, Torino, Sassari and Verona and with information on the FFQ were considered for the present analysis, as the FFQ data was necessary to estimate the flavonoids intake.

## 2.2 Lung Function and Allergologic Tests

Each subject performed spirometry for forced $FEV_1$ and forced vital capacity (FVC), following the American Thoracic Society reproducibility criteria. [49] Quanjer *et al.* equations [50] were used to predict $FEV_1\%$ and the lower limit of normal (LLN) for the $FEV_1/FVC$. Subjects underwent methacholine challenge test if they had $FEV_1/FVC \geq 70\%$ and $\geq$ LLN, following a protocol described elsewhere. [51] Bronchodilator challenge test was performed on participants with a $FEV_1/FVC$ $< 70\%$ or $<$ LLN and, if eligible, they were invited on a second occasion to undergo methacholine challenge test. SPT was used to assess atopy to common allergens (Cupressus arizonica, Dermatophagoides pteronyssinus, Artemisia vulgaris, Dermatophagoides farinae, Ambrosia artemisifolia, Alternaria tenuis, Parietaria judaica, dog dandruff, Corylus avellana, cat fur, Olea europea, Betula verrucosa, Cladosporium herbarum and Phleum pratense). Positive histamine and negative diluent controls were used and, after 20 minutes, the test was considered positive if the wheal diameter was greater than 3 mm. [52]

## 2.3 Dietary intake assessment

The Italian version of the validated European Prospective Investigation into Cancer and Nutrition (EPIC) FFQ, which was based on Italian dietary habits and developed in the frame of an international survey, was used to collect Information on dietary intake. [53] The daily consumption of food items, energy, macro- and micronutrients was calculated using the NAF (Nutritional Analysis of Food Frequency Questionnaires, National Cancer Institute, Milan, Italy) software. [54] The database with the

information on the food composition for epidemiological studies in Italy was used to obtain nutrient data for specific foods. [55] The flavonoids intake was estimated from fruits, vegetables and other plant-derived foods intake contained in the FFQ. A total of $65$ food items, reported in Table 2.1, were considered for the estimation. Total flavonoids and the seven major subclasses intake (flavanones, anthocyanins, flavan-3-ols, flavonols, flavones, polymers and proanthocyanidins) were calculated using the updated and expanded US Department of Agriculture (USDA) flavonoid content of foods and the proanthocyanidin databases, [30, 31] expressed as aglycones (mg/day). The FFQ was filled in by the $43\%$ of the participants in the clinical stage of the study ($1182$ out of $2749$ subjects), then a quality control was performed to exclude subjects with unreliable dietary data by following these steps:

1. Exclusion of the subjects for incomplete FFQ: participants who filled in less than the $80\%$ of the $434$ questions and nested questions of the FFQ ($n = 43$) were excluded.

2. Exclusion of the subjects on the basis of basal metabolic rate (BMR): BMR was estimated both on adults ($\leq 60$ year old) [56] and on elderly subjects ($> 60$ year old) [57] using sex-specific equations. Subjects with missing data needed for BMR estimation were excluded ($n = 24$). Subjects were then excluded if the total energy intake (EI) was too high or too low respect to the BMR estimated. To achieve this, EI:BMR ratio was computed and the subjects with a ratio below the 0.5th sample centile ($n = 4$) or above the 99.5th sample centile ($n = 4$) were excluded.

3. Exclusion of the subjects on the basis of the EI: participants who had extreme low levels ($< 600$ Kcal for women and $< 800$ kcal for men) or extreme high levels ($> 6000$ Kcal for women and $> 8000$ kcal for men) of EI were excluded. Among the subjects, $5$ male and $5$ females were found to have an extreme low EI and no subjects were found to have an EI higher than the threshold.

Table 2.1: List of the 65 food from the European Prospective Investigation into Cancer and Nutrition Food Frequency Questionnaire Food Frequency Questionnaire (EPIC FFQ) considered for the estimation of flavonoids intake.

| No. | Food description |
|---|---|
| **1** | Stuffed Pastas |
| **2** | Vegetal soup |
| **3** | Legume soup |
| **4** | Sliced pizza |
| **5** | Pizza |
| **6** | Home-made pizza |
| **7** | Tomatoes (in season) |
| **8** | Tomatoes (out of season) |
| **9** | Green salad |
| **10** | Raw peppers |
| **11** | Onions |
| **12** | Artichokes and celeries |
| **13** | Raw carrots |
| **14** | Potatoes |
| **15** | Baked beans |
| **16** | Peas |
| **17** | Cooked onions |
| **18** | Cooked carrots |
| **19** | Broccoli |
| **20** | Brussels sprouts |
| **21** | Cauliflower |
| **22** | Turnip tops |
| **23** | Cabbage |
| **24** | Black cabbage |
| **25** | Spinach |
| **26** | Eggplant |
| **27** | Beet |

**Table 2.1 continued from previous page**

| No. | Food description |
|---|---|
| **28** | Mayonnaise and Russian salad |
| **29** | Apple |
| **30** | Pear |
| **31** | Banana |
| **32** | Kiwi |
| **33** | Orange and Grapefruit |
| **34** | Tangerine |
| **35** | Grape |
| **36** | Peach |
| **37** | Apricot |
| **38** | Plum |
| **39** | Strawberry |
| **40** | Melon |
| **41** | Fruit salad |
| **42** | Dried fruits |
| **43** | Nuts |
| **44** | Red wine |
| **45** | White wine |
| **46** | Aperitifs and fortified wines |
| **47** | Beer |
| **48** | Orange juice |
| **49** | Fruit juice |
| **50** | Cappuccino |
| **51** | Coffee with whole milk |
| **52** | Coffee with low-fat milk |
| **53** | Decaffeinated coffee |
| **54** | Coffee (Espresso) |
| **55** | Coffee (Moka pot) |
| **56** | Other types of coffee |

**Table 2.1 continued from previous page**

| No. | Food description |
|---|---|
| **57** | Tea |
| **58** | Yogurt with fruits |
| **59** | Marmalade |
| **60** | Spreadable chocolate |
| **61** | Dairy desserts |
| **62** | Chocolate |
| **63** | Ice cream (in summer season) |
| **64** | Ice cream (in winter season) |
| **65** | Honey |

## 2.4 Identification of Cases and Controls in Clinics

The subjects with information on the clinical visit, on food and nutrient intakes were $1093$ (Figure 2.1). Participants were hierarchically classified as cases or controls on the basis of the following rules:

1. $159$ cases of current asthma (CA): the participants were classified as asthma case if:

   (a) they reported to have had a history of asthma and had asthma-like symptoms or took medicines for asthma in the last year

   (b) if they were in condition (a) plus one of the following: (i) positive methacholine challenge test with a provocative dose of methacholine causing a $20\%$ drop in $FEV_1$ (PD20) $< 1$ mg; (ii) pre-bronchodilator $FEV_1/FVC < 70\%$ or $<$ LLN [50] with a positive reversibility test (i.e. $FEV_1 > 12\%$ and $> 200$ mL after the administration of $400$ $\mu$g of salbutamol); (iii) pre-bronchodilator $FEV_1/FVC < 70\%$ or $<$LLN with a post-bronchodilator $FEV_1/FVC>$ LLN and $> 70\%$ and a post-bronchodilator $FEV_1 > 80\%$ predicted. [50]

2. 78 cases of past asthma (PA): subjects that reported to have had a history of asthma but did not have the full criteria for CA.

3. 10 cases of COPD: subjects with postbronchodilator $FEV_1/FVC < 70\%$ or $<$ LLN without asthma.

4. 47 cases of CB: subjects that were not included in the previous categories (COPD or asthma) and that reported chronic cough or phlegm ($> 3$ months/year for at least 2 years).

5. 167 cases of allergic rhinitis (AR), and 125 cases of non-allergic rhinitis (NAR): subjects that had nasal allergies or nasal problems in the presence of animal(s), pollens, dust plus negative SPT (NAR) or positive SPT to at least one allergen (AR). The information on the nasal symptoms (sneeze, runny nose, itchy nose, congestion, postnasal drip, decreased or absent sense of smell, facial pain or headache) were collected through a questionnaire.

6. 397 controls: subjects who reported not to have any nasal/respiratory symptoms/conditions, both in the clinical questionnaire neither in the clinic and in the screening questionnaire. These subjects were not classified as cases and had both prebronchodilator $FEV_1/FVC >$ LLN and $> 70\%$ and (b) $FEV_1 > 70\%$ predicted.

7. 93 subjects unclassified: due to missing data, these subjects were not classified into any of the categories described above.

The unclassified subjects were excluded from the analyses, as well as the subjects classified as COPD; therefore, the final sample included 990 subjects. In some subjects, there might be an overlay of some diseases, but due to the hierarchical classification, the subjects were included in only one of the categories. Subjects affected by asthma were classified as CA or PA, but they could also be affected by COPD/CB/AR/NAR; subjects affected by CB could present AR/NAR but not asthma and subjects classified as AR or NAR did not present any other disease.

## 2.5 Statistical analyses

The characteristics of the subjects were reported as percentages, median and interquartile range (IQR) (non-normality of data distribution) or means and standard deviation (SD) (normal distribution of data); flavonoids and foods intake were summarized as median and IQR as not normally distributed, and the correlation among total flavonoids and all the subclasses considered were calculated. Differences among groups of cases and controls were tested by using $\chi^2$ test, Kruskall-Wallis and Student t test, as appropriate.

The exposure to flavonoids (total and each subclass) was analyzed in a continuous fashion for a variation of one standard deviation. Flavonoids were also considered in quartiles on the basis of the distribution of the exposure in controls.

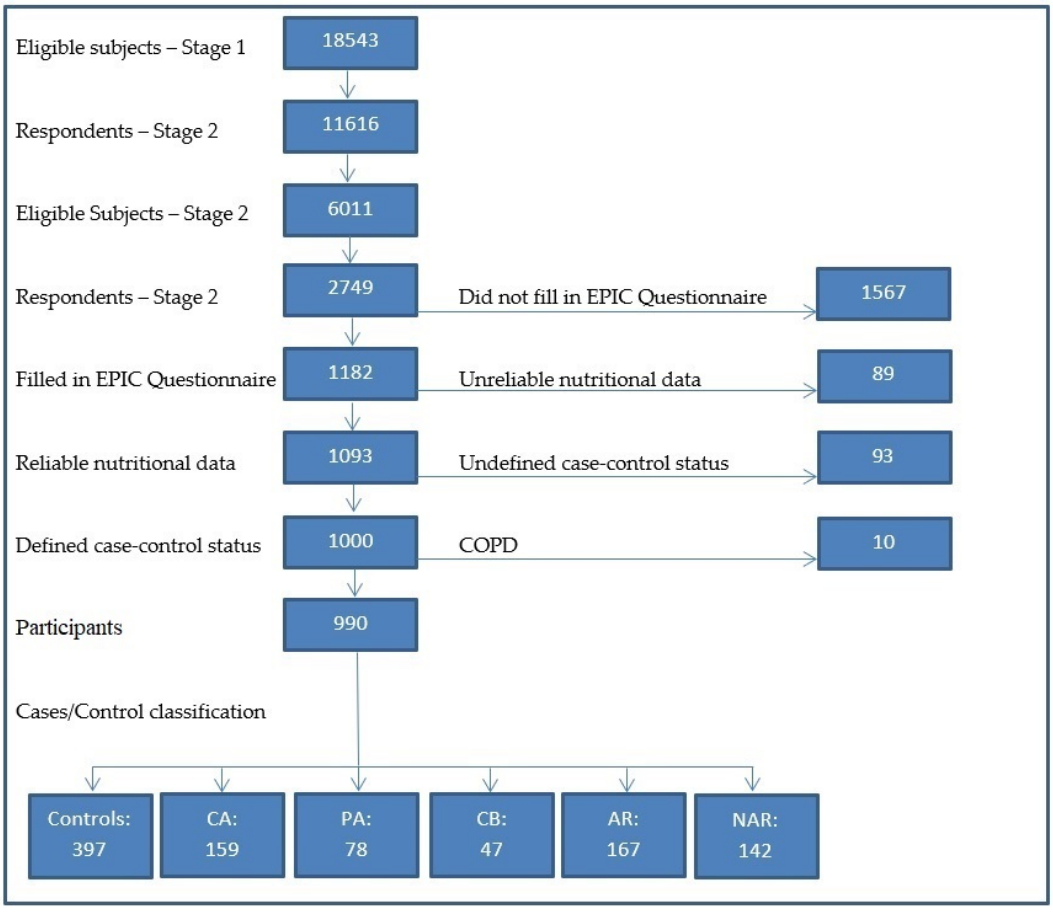



Figure 2.1: Flowchart of the subject selection (CA: current asthma, PA: past asthma, CB: chronic bronchitis, AR: allergic rhinitis, NAR: non-allergic rhinitis). (Source: Mattioli V *et al.*, 2020)

We decided to use these two different approaches as there are some advantages in both. With the use of quartile approach, it is possible to make a comparison between extreme groups of flavonoids intake, reduce the effect of the outliers and detect non-linear associations. Conversely, the continuous approach permits to detect linear associations, if present, even in case of low sample size, moreover it makes easier the comparison with the evidences obtained in other studies. Several multinomial logistic regression models were used to assess the association between flavonoids (total, flavanones, anthocyanins, flavan-3-ols, flavonols, flavones, polymers and proanthocyanidins) and the case-control status, expressed as a 6-level variable (CA, PA, CB, AR, NAR, control). The associations between the exposures and the outcome were reported by relative risk ratios (RRRs) and their $95\%$ Confidence Intervals (CIs), using the group of controls as the reference category. The following potential confounders were included in the models: study sample/cohort (ISAYA, ECRHS-Italy, new random sample), centre (Verona, Pavia, Torino and Sassari), gender, age, body mass index (BMI), education (low = completed before the age of $16$, high = completed after the age of $16$) as a proxy of socioeconomic status, smoking habit (never smoker, past smoker i.e. not smoking in the last month, current smoker), alcohol intake (g/day), total energy intake ($4$ kcal/day), vitamin C (mg/day) and total fruit intake (g/day).

The potential confounders were chosen according the pre-existing knowledge, however other $2$ models with the continuous approach were performed to check for the consistency of the results. According to some studies, BMI could be a mediator in the association between diet and respiratory diseases, thus its presence in the model, could lead to over-adjustment. [58, 59] Therefore, a model without the adjustment for BMI was performed (Model 1). Moreover, being that the main source of vitamin C are fruits, which are the main source of flavonoids as well, analysis excluding vitamin C intake was performed (Model 2).

In order to check the severity of multicollinearity, we estimated the Variance Inflate Factors (VIF) of the models previously described. All the VIF resulted below $5.00$, which is the threshold suggesting the presence of severe multicollinearity. [60] The software STATA 14.2 was used to perform the analyses.

# Chapter 3

# Results

The main characteristics of the participants, age gender and BMI, were homogeneous among the groups of cases and controls; physical activity and educational level were homogeneous as well (Table 3.4). The overall mean age was $50.7 \pm 12.3$, the proportion of males were $48.79\%$ and median BMI was $24.7$ (IQR $22.3; 27.7$). Smoking, drinking habits and alcohol intake were significantly different across the groups of cases and controls (Table 3.4), in particular the number of smokers and drinkers were proportionally higher in the group of subjects affected by chronic bronchitis. ($57.45\%$ smokers and $34.04\%$ drinkers respectively; see Table 3.4 for other details).

Comparing the characteristics of the subjects who fulfilled the EPIC FFQ ($n = 1182$) with the ones of the subjects who did not ($n = 1567$), we have found that in the group of controls, CA and AR, age was significantly higher in subjects who fulfilled the FFQ compared to subjects who did not (see Tables 3.2). The comparison of the characteristics of the subjects classified as case/controls ($n = 990$) or unclassified ($n = 93$) are reported in Table 3.3. The age was significantly higher in the group of unclassified respect to the group of classified, whilst BMI and educational level were both significantly lower in the group of unclassified compared to classified.

The intake of flavonoids, total and in all the subclasses, was consistent across cases and controls (overall median of total flavonoids intake $382.1$ mg/day, IQR $248.8; 529.8$) and the intake of foods rich in flavonoids was homogeneous as well

(Table 3.4). Fruits and vegetable consumption were similar in all groups, with a median intake and interquartile range of $289.1$ $(191.9; 425.5)$ g/day and $130.2$ $(86.7; 194.5)$ g/day respectively. The differences in term of total energy intake and vitamin C intake were not statistically significant across the groups (overall median energy intake $1909.3$, IQR $1505.5; 2398.9$ kcal/day; overall median vitamin C intake $117.6$, IQR $82.6; 161.9$ mg/day). In Table 3.5, the correlation among the groups of flavonoids are reported. The correlations were mostly moderate, excluding strong correlations between total flavonoids, polymers and proanthocyanidins.

The results of the regression analysis are reported in Table 3.6. No significant associations between any of the flavonoid subclasses and risk of chronic respiratory diseases were found in the unadjusted analysis. In the adjusted regression analyses, a significant association between flavanones intake and reduced risk of NAR was found. An intake of $1$ standard deviation of flavanones, which correspond to $26.1$ mg, was associated with a decreased risk of NAR (RRR=$0.68$, $95\%$ CI $0.47; 0.97$; Table 3.6). The result was consistent with that of the quartile approach: comparing the subjects in the highest vs. lowest quartile of flavanone intake, the RRR was $0.24$ ($95\%$ CI $0.10; 0.59$; Table 3.7e).

In the quartile analyses, an increased risk of PA was found comparing the second vs. the first quartile of flavone intake, in both unadjusted and adjusted analyses (adjusted RRR $3.01$, $95\%$ CI $1.37; 6.59$) (Table 3.7b). Comparing the third quartile vs. the first quartile of flavanones intake, unadjusted RRR of having AR was $1.68$ ($95\%$ CI $1.01; 2.81$), but after adjustment the result became not significant (Table 3.7d). Comparing the third vs. the first quartile of intake of polymers, a reduced risk of AR was found (unadjusted RRR $0.56$, $95\%$ CI $0.32; 0.96$), but after adjustment it was no longer significant (Table 3.7d). For the other outcomes considered there was no evidence of significant association with flavonoids intake.

In Table 3.8 and 3.9, the results obtained in the continuous analysis previously reported are compared with the results of the models without the adjustment for BMI (Model 1, Table 3.8) and without the adjustment for vitamin C intake (Model 2, Table 3.9). The results were consistent with the results obtained in the first model, with a significant association between flavanones and reduced risk of NAR.

Table 3.1: General characteristics of the participants. (CA: current asthma, PA: past asthma, CB: chronic bronchitis, AR: allergic rhinitis, NAR: non-allergic rhinitis; $FEV_1$: forced expiratory volume in the 1st second; FVC: forced vital capacity)

| | **Controls** ($n = 397$) | **CA** ($n = 159$) | **PA** ($n = 78$) | **CB** ($n = 47$) | **AR** ($n = 167$) | **NAR** ($n = 142$) | **p-value** |
|---|---|---|---|---|---|---|---|
| **Age at the clinical visit, years (mean, SD)** | 51.9 (12.0) | 50.5 (12.6) | 45.2 (11.3) | 49.1 (13.7) | 49.7 (12.5) | 52.3 (12.0) | 0.726 |
| **Gender (% Male)** | 48.36 | 49.69 | 44.87 | 55.32 | 52.69 | 44.37 | 0.625 |
| **$FEV_1$ (mean, SD)** | 3.3 (0.8) | 3.0 (0.9) | 3.4 (0.8) | 3.3 (1.0) | 3.4 (0.8) | 3.2 (0.8) | 0.136 |
| **FVC (mean, SD)** | 4.0 (1.0) | 4.0 (1.2) | 4.2 (1.0) | 4.1 (1.2) | 4.2 (1.0) | 3.9 (1.0) | 0.086 |
| **Smoking habits (%)** | | | | | | | **0.013** |
| Non-smoker | 53.79 | 50.31 | 57.14 | 44.68 | 57.49 | 43.66 | |
| Ex-smoker | 32.58 | 27.67 | 27.27 | 21.28 | 26.35 | 34.51 | |
| Current smoker | 13.64 | 22.01 | 15.58 | 34.04 | 16.17 | 21.83 | |
| Pack-years (mean, SD) | 21.4 (26.0) | 21.5 (28.2) | 13.7 (15.4) | 32.0 (28.6) | 17.7 (15.1) | 19.1 (21.5) | **<0.001** |
| **Drinking habits (% Current drinker)** | 35.10 | 47.17 | 37.18 | 57.45 | 40.72 | 41.13 | **0.018** |
| **Total alcohol (g/day)** | | | | | | | **0.023** |
| Abstainers | 64.03 | 52.2 | 62.82 | 41.3 | 59.51 | 56.74 | |
| Ex-drinkers | 1.53 | 0.63 | 0 | 2.17 | 1.23 | 2.13 | |
| <5 | 6.63 | 14.47 | 10.26 | 10.87 | 5.52 | 13.48 | |
| 5-15 | 17.6 | 16.98 | 17.95 | 19.57 | 15.95 | 13.48 | |
| 15-30 | 6.12 | 10.06 | 6.41 | 13.04 | 13.5 | 10.64 | |
| 30-120 | 4.08 | 5.66 | 2.56 | 13.04 | 4.29 | 3.55 | |
| **BMI, kg/m$^2$ (median, IQR)** | 25.0 (22.4;27.7) | 24.8 (22.0;27.6) | 23.9 (21.9;27.0) | 25.5 (22.1;28.6) | 24.3 (22.6;27.2) | 24.6 (22.2;28.0) | 0.692 |
| **BMI, kg/m$^2$ (%)** | | | | | | | 0.832 |
| <25 | 50.13 | 50.31 | 58.97 | 47.83 | 57.23 | 52.11 | |
| ≥25 & <30 | 34.76 | 37.74 | 28.21 | 39.13 | 30.72 | 33.80 | |
| ≥30 | 15.11 | 11.95 | 12.82 | 13.04 | 12.05 | 14.08 | |
| **Physical activity (%)** | | | | | | | 0.350 |
| Heavy | 5.79 | 4.4 | 6.41 | 10.64 | 8.98 | 6.34 | |
| Moderate | 36.27 | 35.22 | 32.05 | 27.66 | 41.32 | 42.25 | |
| Light | 57.93 | 60.38 | 61.54 | 61.7 | 49.7 | 51.41 | |
| **Education level (% High)** | 75.95 | 75.95 | 87.18 | 70.21 | 78.44 | 68.79 | 0.056 |

Table 3.2: Main characteristics of subjects participating in the clinical stage of the GEIRD study, according to their participation in the nutritional protocol (with or without EPIC FFQ) and to their case-control status (Controls, CA = current asthma, PA = past asthma, CB = chronic bronchitis, AR = allergic rhinitis, NAR = non-allergic rhinitis; $FEV_1$ = forced expiratory volume in the 1st second; FVC = forced vital capacity).

(a) Controls

| | **Controls with EPIC** ($n = 397$) | **Controls without EPIC** ($n = 415$) | **P-value** |
|---|---|---|---|
| **Age at the clinical visit, years (mean, SD)** | 51.9 (12.0) | 49.7 (12.9) | **0.013** |
| **Gender (% Male)** | 48.36 | 51.33 | 0.399 |
| **$FEV_1$ (mean, SD)** | 3.3 (0.8) | 3.4 (0.8) | 0.381 |
| **FVC (mean, SD)** | 4.0 (1.0) | 4.1 (0.9) | 0.686 |
| **Smoking habits (%)** | | | 0.138 |
| Non-smoker | 53.79 | 50.36 | |
| Ex-smoker | 32.58 | 30.84 | |
| Current smoker | 13.64 | 18.8 | |
| **Pack-years (mean, SD)** | 21.4 (26.0) | 18.4 (30.5) | 0.306 |
| **Drinking habits (% Current drinker)** | 35.1 | 38.98 | 0.253 |
| **Total alcohol (g/day)** | | | 0.497 |
| Abstainers | 64.03 | 61.39 | |
| Ex-drinkers | 1.53 | 0.99 | |
| $<5$ | 6.63 | 8.91 | |
| 5-15 | 17.6 | 18.56 | |
| 15-30 | 6.12 | 7.67 | |
| 30-120 | 4.08 | 2.48 | |
| **BMI, kg/m$^2$ (median, 1st quartile, 3rd quartile)** | 25.0 (22.4;27.7) | 24.9 (21.7;28.0) | 0.906 |
| **BMI, kg/m$^2$ (%)** | | | 0.898 |
| $<25$ | 50.13 | 50.60 | |
| $\geq 25$ & $< 30$ | 34.76 | 35.42 | |
| $\geq 30$ | 15.11 | 13.98 | |
| **Physical activity (%)** | | | 0.458 |
| Heavy | 5.79 | 7.95 | |
| Moderate | 36.27 | 34.46 | |
| Light | 57.93 | 57.59 | |
| **Education level (% High)** | 75.95 | 73.79 | 0.479 |

(b) Current asthma (CA)

| | **CA with EPIC** ($n = 159$) | **CA without EPIC** ($n = 441$) | **P-value** |
|---|---|---|---|
| **Age at the clinical visit, years (mean, SD)** | 50.5 (12.6) | 45.2 (11.8) | **<0.001** |
| **Gender (% Male)** | 49.69 | 50.79 | 0.811 |
| **$FEV_1$ (mean, SD)** | 3.0 (0.9) | 3.2 (0 .9) | **0.007** |
| **FVC (mean, SD)** | 4.0 (1.2) | 4.2 (1.2) | 0.093 |
| **Smoking habits (%)** | | | 0.411 |
| Non-smoker | 50.31 | 45 | |
| Ex-smoker | 27.67 | 28.18 | |
| Current smoker | 22.01 | 26.82 | |
| **Pack-years (mean, SD)** | 21.5 (28.2) | 16.3 (17.6) | 0.059 |
| **Drinking habits (% Current drinker)** | 47.17 | 40.55 | 0.148 |
| **Total alcohol (g/day)** | | | 0.250 |
| Abstainers | 52.2 | 58.92 | |
| Ex-drinkers | 0.63 | 2.35 | |
| <5 | 14.47 | 9.15 | |
| 5-15 | 16.98 | 15.02 | |
| 15-30 | 10.06 | 10.33 | |
| 30-120 | 5.66 | 4.23 | |
| **BMI, kg/m$^2$ (median, 1st quartile, 3rd quartile)** | 24.8 (22.0;27.6) | 24.7 (22.0;27.8) | 0.737 |
| **BMI, kg/m$^2$ (%)** | | | 0.136 |
| <25 | 50.31 | 54.55 | |
| $\geq 25$ & $< 30$ | 37.74 | 29.6 | |
| $\geq 30$ | 11.95 | 15.85 | |
| **Physical activity (%)** | | | 0.712 |
| Heavy | 4.4 | 5.9 | |
| Moderate | 35.22 | 32.88 | |
| Light | 60.38 | 61.22 | |
| **Education level (% High)** | 75.95 | 79.26 | 0.386 |

(c) Past asthma (PA)

| | **PA with EPIC** ($n = 78$) | **PA without EPIC** ($n = 193$) | **P-value** |
|---|---|---|---|
| **Age at the clinical visit, years (mean, SD)** | 45.2 (11.3) | 45.1 (10.4) | 0.958 |
| **Gender (% Male)** | 44.87 | 48.44 | 0.595 |
| **$FEV_1$ (mean, SD)** | 3.4 (0.8) | 3.4 (0.9) | 0.863 |
| **FVC (mean, SD)** | 4.2 (1.0) | 4.2 (1.1) | 0.978 |
| **Smoking habits (%)** | | | 0.773 |
| Non-smoker | 57.14 | 52.36 | |
| Ex-smoker | 27.27 | 29.84 | |
| Current smoker | 15.58 | 17.8 | |
| **Pack-years (mean, SD)** | 13.7 (15.4) | 15.5 (24.8) | 0.716 |
| **Drinking habits (% Current drinker)** | 37.18 | 36.65 | 0.935 |
| **Total alcohol (g/day)** | | | 0.722 |
| Abstainers | 62.82 | 62.9 | |
| Ex-drinkers | 0 | 2.15 | |
| $<5$ | 10.26 | 9.14 | |
| 5-15 | 17.95 | 13.98 | |
| 15-30 | 6.41 | 7.53 | |
| 30-120 | 2.56 | 4.3 | |
| **BMI, kg/m$^2$ (median, 1st quartile, 3rd quartile)** | 23.9 (21.9;27.0) | 24.4 (21.8;27.6) | 0.610 |
| **BMI, kg/m$^2$ (%)** | | | 0.786 |
| $<25$ | 58.97 | 54.75 | |
| $\geq 25$ & $< 30$ | 28.21 | 32.40 | |
| $\geq 30$ | 12.82 | 12.85 | |
| **Physical activity (%)** | | | 0.728 |
| Heavy | 6.41 | 5.76 | |
| Moderate | 32.05 | 37.17 | |
| Light | 61.54 | 57.07 | |
| **Education level (% High)** | 87.18 | 87.23 | 0.990 |

(d) Chronic Bronchitis (CB)

| | **CB with EPIC** ($n = 47$) | **CB without EPIC** ($n = 75$) | **P-value** |
|---|---|---|---|
| **Age at the clinical visit, years (mean, SD)** | 49.1 (13.7) | 50.9 (12.8) | 0.459 |
| **Gender (% Male)** | 55.32 | 48 | 0.431 |
| **$FEV_1$ (mean, SD)** | 3.3 (28.6) | 3.1 (24.9) | 0.270 |
| **FVC (mean, SD)** | 4.1 (1.2) | 4.0 (1.0) | 0.639 |
| **Smoking habits (%)** | | | 0.936 |
| Non-smoker | 44.68 | 41.33 | |
| Ex-smoker | 21.28 | 22.67 | |
| Current smoker | 34.04 | 36 | |
| **Pack-years (mean, SD)** | 32.0 (5.6) | 27.1 (3.8) | 0.460 |
| **Drinking habits (% Current drinker)** | 57.45 | 48.65 | 0.345 |
| **Total alcohol (g/day)** | | | 0.744 |
| Abstainers | 41.3 | 45.95 | |
| Ex-drinkers | 2.17 | 5.41 | |
| $<5$ | 10.87 | 14.86 | |
| 5-15 | 19.57 | 16.22 | |
| 15-30 | 13.04 | 10.81 | |
| 30-120 | 13.04 | 6.76 | |
| **BMI, kg/m$^2$ (median, 1st quartile, 3rd quartile)** | 25.5 (22.1;28.6) | 24.6 (21.8;27.1) | 0.630 |
| **BMI, kg/m$^2$ (%)** | | | 0.799 |
| $<25$ | 47.83 | 53.62 | |
| $\geq 25$ & $< 30$ | 39.13 | 36.23 | |
| $\geq 30$ | 13.04 | 10.14 | |
| **Physical activity (%)** | | | 0.169 |
| Heavy | 10.64 | 2.67 | |
| Moderate | 27.66 | 26.67 | |
| Light | 61.7 | 70.67 | |
| **Education level (% High)** | 70.21 | 66.22 | 0.647 |

(e) Allergic Rhinitis (AR)

| | **AR with EPIC** ($n = 167$) | **AR without EPIC** ($n = 240$) | **P-value** |
|---|---|---|---|
| **Age at the clinical visit, years (mean, SD)** | 49.7 (12.5) | 46.9 (12.7) | **0.031** |
| **Gender (% Male)** | 52.69 | 52.5 | 0.969 |
| **$FEV_1$ (mean, SD)** | 3.4 (0.8) | 3.4 (0.8) | 0.610 |
| **FVC (mean, SD)** | 4.2 (1.0) | 4.1 (1.0) | 0.650 |
| **Smoking habits (%)** | | | 0.843 |
| Non-smoker | 57.49 | 55.46 | |
| Ex-smoker | 26.35 | 28.99 | |
| Current smoker | 16.17 | 15.55 | |
| **Pack-years (mean, SD)** | 17.7 (15.1) | 21.6 (41.1) | 0.451 |
| **Drinking habits (% Current drinker)** | 40.72 | 39.58 | 0.818 |
| **Total alcohol (g/day)** | | | 0.217 |
| Abstainers | 59.51 | 61.02 | |
| Ex-drinkers | 1.23 | 0.42 | |
| $<5$ | 5.52 | 11.44 | |
| 5-15 | 15.95 | 14.83 | |
| 15-30 | 13.5 | 8.47 | |
| 30-120 | 4.29 | 3.81 | |
| **BMI, kg/m$^2$ (median, 1st quartile, 3rd quartile)** | 24.3 (22.6;27.2) | 24.0 (21.8;26.7) | 0.200 |
| **BMI, kg/m$^2$ (%)** | | | 0.371 |
| $<25$ | 57.23 | 57.08 | |
| $\geq 25$ & $< 30$ | 30.72 | 34.76 | |
| $\geq 30$ | 12.05 | 8.15 | |
| **Physical activity (%)** | | | 0.255 |
| Heavy | 8.98 | 5.44 | |
| Moderate | 41.32 | 38.49 | |
| Light | 49.7 | 56.07 | |
| **Education level (% High)** | 78.44 | 83.19 | 0.228 |

(f) Non-Allergic Rhinitis (NAR)

| | **AR with EPIC** ($n = 142$) | **AR without EPIC** ($n = 168$) | **P-value** |
|---|---|---|---|
| **Age at the clinical visit, years (mean, SD)** | 52.3 (12.0) | 52.6 (14.1) | 0.822 |
| **Gender (% Male)** | 44.37 | 41.67 | 0.632 |
| **$FEV_1$ (mean, SD)** | 3.2 (0.8) | 3.0 (0.8) | 0.107 |
| **FVC (mean, SD)** | 3.9 (1.0) | 3.8 (1.0) | 0.275 |
| **Smoking habits (%)** | | | 0.855 |
| Non-smoker | 43.66 | 45.24 | |
| Ex-smoker | 34.51 | 31.55 | |
| Current smoker | 21.83 | 23.21 | |
| **Pack-years (mean, SD)** | 19.1 (21.4) | 22.7 (26.5) | 0.330 |
| **Drinking habits (% Current drinker)** | 41.13 | 30.95 | 0.063 |
| **Total alcohol (g/day)** | | | 0.168 |
| Abstainers | 56.74 | 66.87 | |
| Ex-drinkers | 2.13 | 3.01 | |
| $<5$ | 13.48 | 9.64 | |
| 5-15 | 13.48 | 14.46 | |
| 15-30 | 10.64 | 4.22 | |
| 30-120 | 3.55 | 1.81 | |
| **BMI, kg/m$^2$ (median, 1st quartile, 3rd quartile)** | 24.6 (22.2;28.0) | 24.6 (22.0;27.3) | 0.281 |
| **BMI, kg/m$^2$ (%)** | | | 0.757 |
| $<25$ | 52.11 | 56.36 | |
| $\geq 25$ & $< 30$ | 33.80 | 30.91 | |
| $\geq 30$ | 14.08 | 12.73 | |
| **Physical activity (%)** | | | 0.092 |
| Heavy | 6.34 | 4.76 | |
| Moderate | 42.25 | 31.55 | |
| Light | 51.41 | 63.69 | |
| **Education level (% High)** | 68.79 | 77.98 | 0.068 |

Table 3.3: Main characteristics of subjects according to their inclusion in the present study (classified as case/control or excluded). Subjects included in the present study were classified in one of the following: control, current asthma, past asthma, chronic bronchitis, allergic rhinitis or non-allergic rhinitis. $FEV_1$: forced expiratory volume in the 1st second; FVC: forced vital capacity.

| | **Classified*** ($n = 990$) | **Excluded** ($n = 93$) | **P-value** |
|---|---|---|---|
| **Age at the clinical visit, years (mean, SD)** | 50.7 (12.3) | 58.4 (13.1) | **>0.001** |
| **Gender (% Male)** | 48.79 | 53.76 | 0.359 |
| **$FEV_1$ (mean, SD)** | 3.3 (0.9) | 3.0 (0.8) | **0.005** |
| **FVC (mean, SD)** | 4.1 (1.0) | 3.8 (0.9) | 0.057 |
| **Smoking habits (%)** | | | 0.149 |
| Non-smoker | 52.23 | 41.76 | |
| Ex-smoker | 30.06 | 35.16 | |
| Current smoker | 17.71 | 23.08 | |
| **Pack-years (mean, SD)** | 20.6 (1.1) | 32.3 (4.2) | **0.001** |
| **Drinking habits (% Current drinker)** | 40.08 | 40.22 | 0.980 |
| **Total alcohol (g/day)** | | | 0.287 |
| Abstainers | 56.04 | 59.14 | |
| Ex-drinkers | 4.40 | 1.33 | |
| <5 | 6.59 | 9.19 | |
| 5-15 | 16.48 | 16.75 | |
| 15-30 | 10.99 | 8.99 | |
| 30-120 | 5.49 | 4.60 | |
| **BMI, kg/m$^2$ (median, 1st quartile, 3rd quartile)** | 26.1 (23.3;28.7) | 24.7 (22.3;27.7) | **0.008** |
| **BMI, kg/m$^2$ (%)** | | | 0.096 |
| <25 | 52.23 | 40.86 | |
| ≥25 & <30 | 34.11 | 44.09 | |
| ≥30 | 13.66 | 15.05 | |
| **Physical activity (%)** | | | 0.183 |
| Heavy | 6.46 | 4.35 | |
| Moderate | 37.07 | 29.35 | |
| Light | 56.46 | 66.30 | |
| **Education level (% High)** | 75.96 | 65.56 | **0.029** |

Table 3.4: Distribution of dietary flavonoid intake studied in GEIRD according to Case or Control status (median intake and interquartile range).

| **Flavonoids (mg/day)** | **Controls** ($n = 397$) | **CA** ($n = 159$) | **PA** ($n = 78$) | **CB** ($n = 47$) | **AR** ($n = 167$) | **NAR** ($n = 142$) | **p-value** |
|---|---|---|---|---|---|---|---|
| **Tot. flavonoids** | 381.9 (255.1;526.5) | 388.3 (258.4;554.9) | 380.9 (212.1;517.5) | 374.5 (198.5;515.1) | 365.6 (238.2;545.5) | 394.2 (277.4;518.4) | 0.975 |
| **Flavanones** | 27.3 (14.0;45.8) | 27.1 (12.8;43.3) | 28.1 (11.1;36.5) | 23.2 (12.3;38.7) | 28.0 (18.3;40.3) | 26.7 (13.4;38.5) | 0.754 |
| **Anthocyanins** | 17.3 (10.0;28.4) | 15.7 (8.8;27.7) | 16.6 (10.7;24.8) | 15.4 (6.2;25.4) | 15.9 (10.3;25.7) | 17.8 (9.3;28.0) | 0.923 |
| **Flavan-3-ols** | 45.5 (26.7;71.9) | 46.0 (27.7;75.2) | 48.2 (26.7;72.3) | 42.9 (28.2;85.8) | 46.6 (25.4;73.4) | 47.7 (29.2;80.1) | 0.930 |
| **Flavonols** | 15.9 (10.8;21.7) | 15.1 (10.4;20.0) | 15.1 (10.6;21.7) | 17.0 (10.8;23.8) | 15.2 (9.6;21.7) | 15.8 (10.2;20.7) | 0.858 |
| **Flavones** | 1.9 (1.1;2.9) | 2.0 (1.2;3.2) | 1.9 (1.3;2.8) | 1.9 (1.2;2.7) | 1.9 (1.0;2.8) | 1.9 (1.0;3.0) | 0.944 |
| **Polymers** | 269.4 (168.6;370.6) | 261.3 (168.6;403.2) | 267.7 (138.3;380.5) | 257.7 (146.1;350.9) | 247.7 (157.3;377.4) | 254.6 (174.7;370.0) | 0.976 |
| **Proanthocyanidins** | 293.0 (188.6;417.2) | 295.3 (188.2;461.4) | 293.3 (154.2;420.0) | 292.0 (163.0;405.5) | 279.3 (178.0;432.5) | 289.0 (197.3;422.1) | 0.978 |
| **Foods (g/day)** | | | | | | | |
| **Fruits** | 302.6 (203.0;433.0) | 288.4 (193.3;424.1) | 275.35 (168.8;425.7) | 211.4 (142.6;398.6) | 290.2 (200.1;401.6) | 280.75 (188.6;429.0) | 0.221 |
| **Vegetables** | 129.0 (79.8;195.1) | 118.6 (81.4;190.9) | 150.95 (105.2;232.0) | 152.1 (101.1;196.3) | 128.7 (89.4;182.5) | 131.05 (91.1;195.2) | 0.177 |
| **Chocolate** | 2.7 (0.0;8.6) | 2.9 (0.7;12.8) | 2.9 (0.7;11.4) | 2.9 (1.3;10.3) | 3.7 (0.7;8.6) | 3.7 (0.7;11.4) | 0.115 |
| **Red wine** | 4.2 (0.0;62.5) | 13.9 (0.0;107.1) | 8.1 (0.0;62.5) | 4.2 (0.0;79.4) | 8.3 (0.0;63.5) | 1.9 (0.0;59.5) | 0.473 |
| **White wine** | 1.4 (0.0;26.8) | 2.1 (0.0;41.7) | 3.5 (0.0;35.7) | 0.5 (0.0;26.8) | 1.0 (0.0;26.8) | 1.0 (0.0;26.8) | 0.740 |
| **Tea** | 10.0 (0.0;85.7) | 5.0 (0.0;64.3) | 10.0 (0.0;64.3) | 15.0 (0.0;150) | 15.0 (0.0;85.7) | 10.0 (0.0;150) | 0.517 |
| **Vitamin C (mg/day)** | 116.3 (83.4;162.8) | 116.9 (84.0;156.4) | 129.3 (79.8;167.8) | 105.3 (71.5;151.7) | 119.4 (84.6;164.4) | 118.9 (80.9;165.4) | 0.840 |
| **Tot. energy intake (kcal/day)** | 1876.1 (1463.6;2387.5) | 1832.4 (1518.5;2220.1) | 2147.8 (1643.2;2565.2) | 2125.3 (1524.2;2777.5) | 1964.0 (1578.0;2318.2) | 1847.1 (1500.7;2421.8) | 0.134 |

CA: current asthma, PA: past asthma, CB: chronic bronchitis, AR: allergic rhinitis, NAR: non-allergic rhinitis.

Table 3.5: Correlation matrix for total flavonoids and the main subgroups (flavanones, anthocyanins, flavan-3-ols, flavonols, flavones, polymers and proanthocyanidins).

| **Flavonoids (mg/day)** | **Tot. flavonoids** | **Flavanones** | **Anthocyanins** | **Flavan-3-ols** | **Flavonols** | **Flavones** | **Polymers** | **Proant.** |
|---|---|---|---|---|---|---|---|---|
| **Tot. flavonoids** | 1.000 | | | | | | | |
| **Flavanones** | 0.398 | 1.000 | | | | | | |
| **Anthocyanins** | 0.609 | 0.354 | 1.000 | | | | | |
| **Flavan-3-ols** | 0.753 | 0.152 | 0.308 | 1.000 | | | | |
| **Flavonols** | 0.560 | 0.281 | 0.581 | 0.409 | 1.000 | | | |
| **Flavones** | 0.442 | 0.324 | 0.726 | 0.263 | 0.677 | 1.000 | | |
| **Polymers** | 0.979 | 0.296 | 0.553 | 0.658 | 0.482 | 0.364 | 1.000 | |
| **Proant.** | 0.961 | 0.269 | 0.511 | 0.634 | 0.428 | 0.347 | 0.992 | 1.000 |

Table 3.6: Unadjusted and adjusted* RRR (95% CI) of being a case of CA, PA, CB, AR and NAR rather than a control ($n = 397$) according to the intake of flavonoids. Significant results are in bold.

| **Flavonoid intake (per mg/d SD increase)** | **CA** ($n = 159$) | **PA** ($n = 78$) | **BC** ($n = 47$) | **AR** ($n = 167$) | **NAR** ($n = 142$) |
|---|---|---|---|---|---|
| **Total flavonoids (unadjusted)** | 0.98 (0.81;1.18) | 0.94 (0.73;1.21) | 0.94 (0.69;1.28) | 0.93 (0.77;1.12) | 0.99 (0.82;1.20) |
| **Total flavonoids (adjusted)** | 1.05 (0.80;1.37) | 0.92 (0.65;1.30) | 1.12 (0.72;1.73) | 0.94 (0.72;1.22) | 1.02 (0.77;1.34) |
| **Flavanones (unadjusted)** | 1.01 (0.85;1.20) | 0.80 (0.60;1.07) | 0.81 (0.57;1.16) | 0.98 (0.82;1.17) | 0.86 (0.69;1.06) |
| **Flavanones (adjusted)** | 1.09 (0.81;1.45) | 0.67 (0.42;1.08) | 0.97 (0.52;1.79) | 0.88 (0.66;1.19) | **0.68 (0.47;0.97)** |
| **Anthocyanins (unadjusted)** | 0.93 (0.77;1.12) | 0.93 (0.73;1.19) | 0.92 (0.68;1.26) | 0.95 (0.79;1.13) | 0.96 (0.79;1.15) |
| **Anthocyanins (adjusted)** | 0.83 (0.62;1.11) | 1.14 (0.76;1.71) | 0.97 (0.62;1.53) | 0.93 (0.70;1.25) | 0.98 (0.71;1.35) |
| **Flavan-3-ols (unadjusted)** | 0.98 (0.81;1.19) | 0.98 (0.76;1.27) | 1.05 (0.78;1.41) | 0.93 (0.76;1.13) | 1.04 (0.86;1.26) |
| **Flavan-3-ols (adjusted)** | 0.98 (0.79;1.22) | 0.89 (0.66;1.20) | 1.08 (0.76;1.54) | 0.92 (0.74;1.15) | 1.06 (0.86;1.32) |
| **Flavonols (unadjusted)** | 0.88 (0.72;1.07) | 0.93 (0.72;1.20) | 1.20 (0.92;1.56) | 0.93 (0.77;1.12) | 0.97 (0.80;1.18) |
| **Flavonols (adjusted)** | 0.81 (0.63;1.06) | 0.98 (0.71;1.36) | 1.37 (0.97;1.94) | 0.88 (0.69;1.13) | 0.94 (0.73;1.21) |
| **Flavones (unadjusted)** | 1.04 (0.87;1.24) | 0.94 (0.73;1.21) | 1.14 (0.86;1.49) | 0.94 (0.78;1.13) | 0.96 (0.79;1.16) |
| **Flavones (adjusted)** | 0.93 (0.71;1.22) | 1.03 (0.71;1.51) | 1.00 (0.69;1.47) | 0.81 (0.62;1.07) | 0.95 (0.71;1.27) |
| **Polymers (unadjusted)** | 0.98 (0.82;1.18) | 0.96 (0.75;1.23) | 0.93 (0.67;1.27) | 0.93 (0.77;1.12) | 1.01 (0.83;1.22) |
| **Polymers (adjusted)** | 1.08 (0.84;1.39) | 0.97 (0.70;1.33) | 1.09 (0.72;1.66) | 0.98 (0.76;1.26) | 1.03 (0.80;1.34) |
| **Proanthocyanidins (unadjusted)** | 1.02 (0.85;1.22) | 0.96 (0.75;1.24) | 0.95 (0.69;1.30) | 0.95 (0.79;1.15) | 1.01 (0.83;1.22) |
| **Proanthocyanidins (adjusted)** | 1.11 (0.88;1.41) | 0.95 (0.69;1.29) | 1.05 (0.70;1.58) | 1.00 (0.78;1.26) | 1.03 (0.81;1.32) |

CA: current asthma, PA: past asthma, CB: chronic bronchitis, AR: allergic rhinitis, NAR: non-allergic rhinitis.

RRR: Relative risk ratio, CI: confidence interval.

*The estimates were adjusted for: age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, educational level, total fruit intake, vitamin C intake, total energy intake.

Table 3.7: Unadjusted and adjusted* RRR (and 95% CI) to be a case of current asthma (CA, $n = 159$), past asthma (PA, $n = 78$), chronic bronchitis (CB, $n = 47$), allergic rhinitis (AR, $n = 167$) or non-allergic rhinitis (NAR, $n = 142$) rather than a control ($n = 397$), according to quartiles of flavonoids intake (based on the distribution of controls).

(a) Current asthma (CA)

| | Quartile of intake | | | | |
|---|---|---|---|---|---|
| **CA** | **1** | **2** | **3** | **4** | **p-value (trend)** |
| **Total flavonoids** | 1.00 | 1.04 (0.61;1.75) | 1.01 (0.60;1.71) | 1.17 (0.70;1.96) | 0.588 |
| **Total flavonoids (adjusted)** | 1.00 | 1.17 (0.66;2.06) | 1.19 (0.65;2.19) | 1.54 (0.78;3.04) | 0.244 |
| **Flavanones** | 1.00 | 0.96 (0.57;1.61) | 1.06 (0.64;1.76) | 0.89 (0.52;1.50) | 0.761 |
| **Flavanones (adjusted)** | 1.00 | 1.03 (0.60;1.79) | 1.24 (0.69;2.25) | 1.01 (0.47;2.18) | 0.770 |
| **Anthocyanins** | 1.00 | 0.97 (0.59;1.59) | 0.66 (0.38;1.13) | 0.86 (0.51;1.42) | 0.308 |
| **Anthocyanins (adjusted)** | 1.00 | 0.92 (0.55;1.56) | 0.56 (0.30;1.03) | 0.69 (0.33;1.44) | 0.142 |
| **Flavan-3-ols** | 1.00 | 1.06 (0.63;1.80) | 0.93 (0.54;1.59) | 1.22 (0.73;2.04) | 0.550 |
| **Flavan-3-ols (adjusted)** | 1.00 | 1.08 (0.62;1.89) | 0.98 (0.55;1.73) | 1.26 (0.69;2.30) | 0.551 |
| **Flavonols** | 1.00 | 0.94 (0.57;1.56) | 0.90 (0.54;1.50) | 0.80 (0.48;1.36) | 0.411 |
| **Flavonols (adjusted)** | 1.00 | 0.96 (0.56;1.64) | 0.82 (0.46;1.48) | 0.76 (0.40;1.47) | 0.384 |
| **Flavones** | 1.00 | 1.24 (0.73;2.10) | 1.04 (0.60;1.79) | 1.30 (0.77;2.19) | 0.465 |
| **Flavones (adjusted)** | 1.00 | 1.33 (0.76;2.32) | 0.99 (0.55;1.81) | 1.22 (0.61;2.46) | 0.818 |
| **Polymers** | 1.00 | 1.09 (0.65;1.81) | 0.78 (0.45;1.35) | 1.14 (0.68;1.89) | 0.901 |
| **Polymers (adjusted)** | 1.00 | 1.20 (0.69;2.08) | 0.92 (0.50;1.69) | 1.42 (0.74;2.71) | 0.467 |
| **Proanthocyanidins** | 1.00 | 0.98 (0.58;1.66) | 0.71 (0.41;1.23) | 1.31 (0.80;2.16) | 0.440 |
| **Proanthocyanidins (adjusted)** | 1.00 | 1.09 (0.63;1.90) | 0.84 (0.45;1.56) | 1.60 (0.86;2.96) | 0.199 |

RRR: Relative risk ratio; CI: confidence interval.

*The estimates were adjusted for: age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, educational level, total fruit intake, vitamin C intake, total energy intake.

(b) Past asthma (PA)

| | Quartile of intake | | | | |
|---|---|---|---|---|---|
| **PA** | **1** | **2** | **3** | **4** | **p-value (trend)** |
| **Total flavonoids** | 1.00 | 0.61 (0.30;1.22) | 0.85 (0.45;1.61) | 0.69 (0.35;1.35) | 0.424 |
| **Total flavonoids (adjusted)** | 1.00 | 0.63 (0.30;1.33) | 0.88 (0.41;1.87) | 0.61 (0.25;1.50) | 0.423 |
| **Flavanones** | 1.00 | 0.55 (0.26;1.14) | 1.09 (0.59;2.04) | 0.63 (0.31;1.27) | 0.520 |
| **Flavanones (adjusted)** | 1.00 | 0.64 (0.30;1.40) | 1.35 (0.64;2.85) | 0.77 (0.27;2.18) | 0.882 |
| **Anthocyanins** | 1.00 | 1.43 (0.72;2.82) | 1.43 (0.72;2.82) | 0.77 (0.36;1.67) | 0.595 |
| **Anthocyanins (adjusted)** | 1.00 | 1.42 (0.69;2.94) | 1.69 (0.76;3.76) | 1.11 (0.38;3.23) | 0.575 |
| **Flavan-3-ols** | 1.00 | 0.86 (0.42;1.74) | 1.06 (0.54;2.08) | 1.01 (0.51;1.99) | 0.834 |
| **Flavan-3-ols (adjusted)** | 1.00 | 0.83 (0.39;1.76) | 1.00 (0.48;2.07) | 0.76 (0.34;1.68) | 0.625 |
| **Flavonols** | 1.00 | 1.16 (0.60;2.25) | 0.86 (0.42;1.74) | 0.91 (0.45;1.82) | 0.602 |
| **Flavonols (adjusted)** | 1.00 | 1.28 (0.63;2.59) | 0.98 (0.44;2.18) | 1.17 (0.49;2.80) | 0.883 |
| **Flavones** | 1.00 | **2.53 (1.22;5.21)** | 1.68 (0.78;3.63) | 1.35 (0.61;2.99) | 0.904 |
| **Flavones (adjusted)** | 1.00 | **3.01 (1.37;6.59)** | 1.97 (0.83;4.70) | 1.97 (0.69;5.60) | 0.414 |
| **Polymers** | 1.00 | 0.75 (0.38;1.48) | 0.79 (0.40;1.55) | 0.88 (0.45;1.70) | 0.735 |
| **Polymers (adjusted)** | 1.00 | 0.85 (0.41;1.77) | 0.87 (0.40;1.89) | 0.93 (0.40;2.14) | 0.868 |
| **Proanthocyanidins** | 1.00 | 0.63 (0.31;1.27) | 0.80 (0.41;1.55) | 0.84 (0.44;1.62) | 0.736 |
| **Proanthocyanidins (adjusted)** | 1.00 | 0.71 (0.34;1.50) | 0.92 (0.43;1.98) | 0.86 (0.38;1.93) | 0.842 |

RRR: Relative risk ratio; CI: confidence interval.

*The estimates were adjusted for: age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, educational level, total fruit intake, vitamin C intake, total energy intake.

(c) Chronic Bronchitis (CB)

| | Quartile of intake | | | | |
|---|---|---|---|---|---|
| **CB** | **1** | **2** | **3** | **4** | **p-value (trend)** |
| **Total flavonoids** | 1.00 | 0.72 (0.31;1.70) | 0.87 (0.38;1.96) | 0.79 (0.34;1.83) | 0.684 |
| **Total flavonoids (adjusted)** | 1.00 | 0.66 (0.25;1.72) | 0.91 (0.34;2.41) | 1.06 (0.35;3.20) | 0.801 |
| **Flavanones** | 1.00 | 1.24 (0.57;2.72) | 0.70 (0.29;1.71) | 0.70 (0.29;1.71) | 0.235 |
| **Flavanones (adjusted)** | 1.00 | 1.38 (0.59;3.27) | 0.76 (0.25;2.27) | 1.01 (0.27;3.78) | 0.755 |
| **Anthocyanins** | 1.00 | 0.61 (0.25;1.45) | 0.81 (0.36;1.81) | 0.74 (0.32;1.69) | 0.592 |
| **Anthocyanins (adjusted)** | 1.00 | 0.63 (0.25;1.60) | 0.83 (0.32;2.16) | 0.98 (0.29;3.29) | 0.962 |
| **Flavan-3-ols** | 1.00 | 1.47 (0.65;3.32) | 0.64 (0.24;1.73) | 1.19 (0.51;2.79) | 0.872 |
| **Flavan-3-ols (adjusted)** | 1.00 | 1.27 (0.52;3.06) | 0.59 (0.20;1.68) | 1.07 (0.39;2.98) | 0.707 |
| **Flavonols** | 1.00 | 1.31 (0.55;3.13) | 1.11 (0.45;2.73) | 1.31 (0.55;3.13) | 0.654 |
| **Flavonols (adjusted)** | 1.00 | 1.50 (0.60;3.77) | 1.13 (0.40;3.18) | 1.76 (0.59;5.23) | 0.431 |
| **Flavones** | 1.00 | 2.02 (0.83;4.93) | 1.77 (0.71;4.40) | 1.14 (0.42;3.06) | 0.933 |
| **Flavones (adjusted)** | 1.00 | 2.36 (0.90;6.20) | 1.71 (0.61;4.78) | 0.70 (0.20;2.51) | 0.511 |
| **Polymers** | 1.00 | 0.57 (0.24;1.35) | 0.69 (0.31;1.57) | 0.69 (0.31;1.57) | 0.443 |
| **Polymers (adjusted)** | 1.00 | 0.52 (0.20;1.37) | 0.70 (0.27;1.81) | 0.80 (0.28;2.32) | 0.788 |
| **Proanthocyanidins** | 1.00 | 0.51 (0.21;1.23) | 0.76 (0.34;1.68) | 0.69 (0.31;1.57) | 0.518 |
| **Proanthocyanidins (adjusted)** | 1.00 | 0.47 (0.17;1.25) | 0.75 (0.29;1.91) | 0.71 (0.26;1.97) | 0.673 |

RRR: Relative risk ratio; CI: confidence interval.

*The estimates were adjusted for: age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, educational level, total fruit intake, vitamin C intake, total energy intake.

(d) Allergic Rhinitis (AR)

| | Quartile of intake | | | | |
|---|---|---|---|---|---|
| **AR** | **1** | **2** | **3** | **4** | **p-value (trend)** |
| **Total flavonoids** | 1.00 | 0.90 (0.54;1.49) | 0.77 (0.46;1.29) | 0.99 (0.60;1.62) | 0.823 |
| **Total flavonoids (adjusted)** | 1.00 | 0.93 (0.54;1.60) | 0.80 (0.44;1.46) | 1.08 (0.56;2.10) | 0.944 |
| **Flavanones** | 1.00 | 1.44 (0.85;2.43) | **1.68 (1.01;2.81)** | 0.98 (0.56;1.71) | 0.851 |
| **Flavanones (adjusted)** | 1.00 | 1.50 (0.86;2.61) | 1.71 (0.94;3.08) | 0.87 (0.40;1.92) | 0.841 |
| **Anthocyanins** | 1.00 | 1.45 (0.88;2.38) | 0.98 (0.58;1.67) | 0.88 (0.51;1.51) | 0.354 |
| **Anthocyanins (adjusted)** | 1.00 | 1.39 (0.82;2.33) | 0.90 (0.49;1.64) | 0.82 (0.39;1.72) | 0.414 |
| **Flavan-3-ols** | 1.00 | 0.85 (0.51;1.43) | 0.96 (0.58;1.60) | 1.01 (0.61;1.67) | 0.858 |
| **Flavan-3-ols (adjusted)** | 1.00 | 0.87 (0.50;1.50) | 0.98 (0.57;1.68) | 1.06 (0.59;1.89) | 0.749 |
| **Flavonols** | 1.00 | 0.64 (0.38;1.06) | 0.71 (0.43;1.17) | 0.77 (0.47;1.25) | 0.333 |
| **Flavonols (adjusted)** | 1.00 | 0.62 (0.36;1.05) | 0.63 (0.36;1.11) | 0.69 (0.37;1.28) | 0.236 |
| **Flavones** | 1.00 | 0.74 (0.44;1.23) | 0.93 (0.56;1.52) | 0.84 (0.51;1.39) | 0.688 |
| **Flavones (adjusted)** | 1.00 | 0.74 (0.43;1.27) | 0.80 (0.46;1.39) | 0.67 (0.34;1.32) | 0.295 |
| **Polymers** | 1.00 | 0.95 (0.58;1.55) | **0.56 (0.32;0.96)** | 0.93 (0.57;1.52) | 0.395 |
| **Polymers (adjusted)** | 1.00 | 0.94 (0.55;1.58) | 0.59 (0.32;1.07) | 0.99 (0.53;1.85) | 0.659 |
| **Proanthocyanidins** | 1.00 | 1.05 (0.64;1.73) | 0.70 (0.41;1.19) | 0.99 (0.60;1.63) | 0.612 |
| **Proanthocyanidins (adjusted)** | 1.00 | 1.05 (0.62;1.77) | 0.76 (0.42;1.39) | 1.08 (0.58;1.99) | 0.968 |

RRR: Relative risk ratio; CI: confidence interval.

*The estimates were adjusted for: age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, educational level, total fruit intake, vitamin C intake, total energy intake.

(e) Non-Allergic Rhinitis (NAR)

| | Quartile of intake | | | | |
|---|---|---|---|---|---|
| **NAR** | **1** | **2** | **3** | **4** | **p-value (trend)** |
| **Total flavonoids** | 1.00 | 1.28 (0.74;2.23) | 1.31 (0.76;2.28) | 1.18 (0.67;2.07) | 0.585 |
| **Total flavonoids (adjusted)** | 1.00 | 1.37 (0.75;2.49) | 1.48 (0.78;2.80) | 1.43 (0.69;2.99) | 0.348 |
| **Flavanones** | 1.00 | 1.04 (0.61;1.76) | 1.39 (0.84;2.31) | **0.44 (0.23;0.84)** | 0.103 |
| **Flavanones (adjusted)** | 1.00 | 0.91 (0.52;1.60) | 1.08 (0.59;1.96) | **0.24 (0.10;0.59)** | **0.039** |
| **Anthocyanins** | 1.00 | 0.71 (0.41;1.23) | 0.98 (0.58;1.66) | 0.88 (0.52;1.50) | 0.924 |
| **Anthocyanins (adjusted)** | 1.00 | 0.67 (0.37;1.20) | 0.98 (0.53;1.81) | 1.02 (0.46;2.25) | 0.819 |
| **Flavan-3-ols** | 1.00 | 1.01 (0.58;1.76) | 0.98 (0.56;1.71) | 1.35 (0.79;2.29) | 0.293 |
| **Flavan-3-ols (adjusted)** | 1.00 | 1.05 (0.58;1.89) | 1.14 (0.63;2.08) | 1.59 (0.85;2.97) | 0.133 |
| **Flavonols** | 1.00 | 0.70 (0.40;1.21) | 0.87 (0.51;1.46) | 0.84 (0.50;1.43) | 0.681 |
| **Flavonols (adjusted)** | 1.00 | 0.71 (0.40;1.27) | 0.86 (0.47;1.55) | 0.79 (0.41;1.53) | 0.610 |
| **Flavones** | 1.00 | 0.90 (0.53;1.55) | 0.88 (0.51;1.51) | 0.98 (0.58;1.67) | 0.924 |
| **Flavones (adjusted)** | 1.00 | 0.97 (0.54;1.72) | 0.94 (0.51;1.72) | 1.07 (0.52;2.21) | 0.917 |
| **Polymers** | 1.00 | 1.43 (0.84;2.45) | 1.04 (0.59;1.84) | 1.14 (0.65;1.99) | 0.973 |
| **Polymers (adjusted)** | 1.00 | 1.41 (0.79;2.52) | 1.05 (0.56;1.97) | 1.19 (0.59;2.41) | 0.903 |
| **Proanthocyanidins** | 1.00 | 1.65 (0.95;2.86) | 1.27 (0.71;2.26) | 1.38 (0.78;2.44) | 0.504 |
| **Proanthocyanidins (adjusted)** | 1.00 | 1.05 (0.62;1.77) | 0.76 (0.42;1.39) | 1.08 (0.58;1.99) | 0.386 |

RRR: Relative risk ratio; CI: confidence interval.

*The estimates were adjusted for: age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, educational level, total fruit intake, vitamin C intake, total energy intake.

Table 3.8: Adjusted RRR (95% CI) of being a case of CA, PA, CB, AR and NAR rather than a control ($n = 397$) according to the intake of flavonoids. In Model 1, BMI was excluded. Under Model 1, the other model with all the potential confounders, used for the analyses of the present work (here is reported for an easier comparison with Model 1). Significant results are in bold.

| **Flavonoid intake (per mg/d SD increase)** | **CA** ($n = 159$) | **PA** ($n = 78$) | **BC** ($n = 47$) | **AR** ($n = 167$) | **NAR** ($n = 142$) |
|---|---|---|---|---|---|
| **Tot. flavonoids (Model 1)** | 1.01 (0.79;1.28) | 0.88 (0.64;1.22) | 0.98 (0.65;1.47) | 0.89 (0.70;1.14) | 1.01 (0.79;1.29) |
| **Tot. flavonoids (all confounders)** | 1.05 (0.80;1.37) | 0.92 (0.65;1.30) | 1.12 (0.72;1.73) | 0.94 (0.72;1.22) | 1.02 (0.77;1.34) |
| **Flavanones (Model 1)** | 1.06 (0.80;1.41) | 0.66 (0.42;1.04) | 0.84 (0.47;1.51) | 0.86 (0.64;1.14) | **0.69 (0.49;0.98)** |
| **Flavanones (all confounders)** | 1.09 (0.81;1.45) | 0.67 (0.42;1.08) | 0.97 (0.52;1.79) | 0.88 (0.66;1.19) | **0.68 (0.47;0.97)** |
| **Anthocyanins (Model 1)** | 0.82 (0.62;1.09) | 1.09 (0.74;1.60) | 0.90 (0.58;1.39) | 0.90 (0.68;1.19) | 0.98 (0.72;1.33) |
| **Anthocyanins (all confounders)** | 0.83 (0.62;1.11) | 1.14 (0.76;1.71) | 0.97 (0.62;1.53) | 0.93 (0.70;1.25) | 0.98 (0.71;1.35) |
| **Flavan-3-ols (Model 1)** | 0.97 (0.78;1.21) | 0.87 (0.65;1.18) | 1.04 (0.73;1.49) | 0.90 (0.72;1.13) | 1.06 (0.85;1.31) |
| **Flavan-3-ols (all confounders)** | 0.98 (0.79;1.22) | 0.89 (0.66;1.20) | 1.08 (0.76;1.54) | 0.92 (0.74;1.15) | 1.06 (0.86;1.32) |
| **Flavonols (Model 1)** | 0.81 (0.63;1.05) | 0.97 (0.71;1.34) | 1.36 (0.96;1.92) | 0.88 (0.69;1.12) | 0.94 (0.73;1.21) |
| **Flavonols (all confounders)** | 0.81 (0.63;1.06) | 0.98 (0.71;1.36) | 1.37 (0.97;1.94) | 0.88 (0.69;1.13) | 0.94 (0.73;1.21) |
| **Flavones (Model 1)** | 0.94 (0.72;1.23) | 1.06 (0.73;1.54) | 1.03 (0.71;1.50) | 0.83 (0.63;1.09) | 0.95 (0.71;1.28) |
| **Flavones (all confounders)** | 0.93 (0.71;1.22) | 1.03 (0.71;1.51) | 1.00 (0.69;1.47) | 0.81 (0.62;1.07) | 0.95 (0.71;1.27) |
| **Polymers (Model 1)** | 1.03 (0.82;1.29) | 0.92 (0.68;1.24) | 0.96 (0.66;1.42) | 0.93 (0.74;1.16) | 1.02 (0.81;1.29) |
| **Polymers (all confounders)** | 1.08 (0.84;1.39) | 0.97 (0.70;1.33) | 1.09 (0.72;1.66) | 0.98 (0.76;1.26) | 1.03 (0.80;1.34) |
| **Proanthocyanidins (Model 1)** | 1.07 (0.86;1.33) | 0.91 (0.68;1.22) | 0.95 (0.65;1.40) | 0.95 (0.76;1.18) | 1.02 (0.81;1.28) |
| **Proanthocyanidins (all confounders)** | 1.11 (0.88;1.41) | 0.95 (0.69;1.29) | 1.05 (0.70;1.58) | 1.00 (0.78;1.26) | 1.03 (0.81;1.32) |

CA: current asthma, PA: past asthma, CB: chronic bronchitis, AR: allergic rhinitis, NAR: non-allergic rhinitis.
RRR: Relative risk ratio, CI: confidence interval.
Model 1 was adjusted for: age, gender, centre, study cohort, smoking habits, alcohol intake, educational level, total fruit intake, vitamin C, total energy intake.
Model with all confounders: Model 1 + BMI.

Table 3.9: Adjusted RRR (95% CI) of being a case of CA, PA, CB, AR and NAR rather than a control ($n = 397$) according to the intake of flavonoids. In Model 1, Vitamin C intake was excluded. Under Model 2, the other model with all the potential confounders, used for the analyses of the present work (here is reported for an easier comparison with Model 2). Significant results are in bold.

| **Flavonoid intake (per mg/d SD increase)** | **CA** ($n = 159$) | **PA** ($n = 78$) | **BC** ($n = 47$) | **AR** ($n = 167$) | **NAR** ($n = 142$) |
|---|---|---|---|---|---|
| **Tot. flavonoids (Model 2)** | 1.04 (0.80;1.37) | 0.92 (0.65;1.30) | 1.12 (0.72;1.73) | 0.93 (0.71;1.21) | 1.02 (0.78;1.34) |
| **Tot. flavonoids (all confounders)** | 1.05 (0.80;1.37) | 0.92 (0.65;1.30) | 1.12 (0.72;1.73) | 0.94 (0.72;1.22) | 1.02 (0.77;1.34) |
| **Flavanones (Model 2)** | 1.10 (0.84;1.43) | 0.75 (0.49;1.15) | 0.98 (0.56;1.73) | 0.97 (0.74;1.27) | **0.70 (0.51;0.98)** |
| **Flavanones (all confounders)** | 1.09 (0.81;1.45) | 0.67 (0.42;1.08) | 0.97 (0.52;1.79) | 0.88 (0.66;1.19) | **0.68 (0.47;0.97)** |
| **Anthocyanins (Model 2)** | 0.85 (0.64;1.13) | 1.16 (0.79;1.71) | 0.98 (0.63;1.54) | 0.98 (0.74;1.31) | 0.97 (0.72;1.31) |
| **Anthocyanins (all confounders)** | 0.83 (0.62;1.11) | 1.14 (0.76;1.71) | 0.97 (0.62;1.53) | 0.93 (0.70;1.25) | 0.98 (0.71;1.35) |
| **Flavan-3-ols (Model 2)** | 0.98 (0.79;1.22) | 0.89 (0.66;1.20) | 1.08 (0.76;1.54) | 0.92 (0.73;1.15) | 1.06 (0.85;1.31) |
| **Flavan-3-ols (all confounders)** | 0.98 (0.79;1.22) | 0.89 (0.66;1.20) | 1.08 (0.76;1.54) | 0.92 (0.74;1.15) | 1.06 (0.86;1.32) |
| **Flavonols (Model 2)** | 0.83 (0.64;1.07) | 1.01 (0.74;1.37) | 1.31 (0.95;1.80) | 0.93 (0.73;1.17) | 0.93 (0.73;1.19) |
| **Flavonols (all confounders)** | 0.81 (0.63;1.06) | 0.98 (0.71;1.36) | 1.37 (0.97;1.94) | 0.88 (0.69;1.13) | 0.94 (0.73;1.21) |
| **Flavones (Model 2)** | 0.96 (0.75;1.23) | 1.06 (0.75;1.50) | 1.02 (0.71;1.46) | 0.89 (0.69;1.16) | 0.94 (0.71;1.23) |
| **Flavones (all confounders)** | 0.93 (0.71;1.22) | 1.03 (0.71;1.51) | 1.00 (0.69;1.47) | 0.81 (0.62;1.07) | 0.95 (0.71;1.27) |
| **Polymers (Model 2)** | 1.07 (0.83;1.37) | 0.96 (0.69;1.32) | 1.09 (0.72;1.64) | 0.95 (0.74;1.22) | 1.04 (0.81;1.34) |
| **Polymers (all confounders)** | 1.08 (0.84;1.39) | 0.97 (0.70;1.33) | 1.09 (0.72;1.66) | 0.98 (0.76;1.26) | 1.03 (0.80;1.34) |
| **Proanthocyanidins (Model 2)** | 1.10 (0.87;1.39) | 0.94 (0.69;1.28) | 1.05 (0.70;1.56) | 0.97 (0.77;1.23) | 1.04 (0.81;1.33) |
| **Proanthocyanidins (all confounders)** | 1.11 (0.88;1.41) | 0.95 (0.69;1.29) | 1.05 (0.70;1.58) | 1.00 (0.78;1.26) | 1.03 (0.81;1.32) |

CA: current asthma, PA: past asthma, CB: chronic bronchitis, AR: allergic rhinitis, NAR: non-allergic rhinitis.
RRR: Relative risk ratio, CI: confidence interval.
Model 2 was adjusted for: age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, educational level, total fruit intake, total energy intake.
Model with all confounders: Model 2 + vitamin C.

# Chapter 4

# Discussion

In this analysis, performed on a multi-case control study on Italian adults, we evaluated the association between flavonoids (total and main subcategories) and respiratory diseases and we found that flavanones are associated with a decreased risk of NAR. The analyses were performed considering flavanones both as a continuous exposure and categorized as quartiles, and in both approaches the association was statistically significant.

Previous studies showed that flavonoids have anti-inflammatory and antioxidant properties. [28, 29, 32–36] Oxidative stress causes airway inflammation, [37] which is known to be involved in the biological mechanisms and worsening of pulmonary diseases. [61, 62] Airway inflammation play a crucial role in all the diseases considered in our study (asthma, chronic bronchitis and rhinitis). [23] In asthma, both small and large airways can be damaged by inflammation, depending on the severity of the disease. [61] In a similar way, the lumen and the wall of the airways can be affected by inflammation in chronic bronchitis. [11]

Some environmental factors can contribute to increase the oxidative stress and inflammation in lungs, and according to experimental studies flavonoids can counteract these effects. Among all flavonoids, quercetin has been widely investigated and it has been shown that it can decrease superoxides and nitric oxide radicals, which in turn are directly involved in the mechanisms of inflammation. [63, 64] Arachidonic acid, which contribute to the production of reactive oxygen species, can be inhibited by flavonoids. [34, 65] Moreover, flavonoids seem to counteract

the adhesion of inflammatory cells as well. [37]

Although experimental studies provide evidence on the beneficial effect of flavonoids on lung health, epidemiological evidence is still scant and unclear. Epidemiological studies reported mixed results and this is might be due to the limited classes of flavonoids analyzed. [66] A diet rich in foods that contain flavonoids (mainly fruits, vegetables, wine and tea) seems to reduce the risk of asthma, according to several population-based studies [67, 68] but this evidence has not been confirmed by other studies. [69] The association between flavonoids and respiratory diseases has been examined only by few population-based studies and the results were inconclusive. According to a study conducted in a population of Dutch adults by Tabak and colleagues, a higher consumption of catechins was associated with a reduced risk of asthma and COPD. [70] In a population-based case-control study conducted in London by Garcia et al, the association between three classes of flavonoids and chronic respiratory diseases (asthma and chronic bronchitis) was studied, but no evidence of a beneficial effect was found. [43] In a clinical trial on the supplementary use soy isoflavone in adults and children with poor asthma control no beneficial effect was found on lung function. [71]

According to a recent hypothesis, microbiota might be involved in the protection against inflammation-mediated airways diseases. This theory has been investigated in a small sample of $23$ allergic subjects where they found that a high intake of phenolic compounds is associated with a greater stability of the gut microbiota, which in turn might contribute to reduce inflammation. [72] In a recent cohort study in subjects with stable asthma it has been reported that soy genistein intake is associated with a better lung function and asthma control. [73] Until now, there is only a little evidence from trials on humans. In an intervention on $42$ subjects affected by asthma it has been found that a passion fruit puree extract rich in bioflavonoids decrease the severity of asthma symptoms, [74] whilst in a recent randomized controlled trial no evidence of a beneficial effect of supplementation with soy genistein on asthma or lung function was found in adults with unstable asthma. [71]

In the present population-based study only a limited evidence of the association between flavonoids and respiratory diseases was found. The subjects considered

were affected by asthma, chronic bronchitis or allergic rhinitis in stable condition. The protective effect of flavonoids probably occurs mainly in subjects with an exacerbated inflammation, which is not characteristic of stable patients, and this might explain this lack of evidence.

In this study, an association between flavanones and a reduced risk of non-allergic rhinitis was found. The SPT, which was used to assess atopy on subjects affected by rhinitis, detect the systemic presence of serum allergen-specific IgE (immunoglobulin E) in allergic subjects. Nevertheless, it has been demonstrated that allergen-specific IgE can be found also in subject affected by non-allergic rhinitis, but only locally in nasal mucosa, so its presence is not detectable by SPT. [75] It is believed that flavonoids are able to inhibit the formation of IgE [33, 41] and this might at least partially explain the protective effect found in our analyses on patients affected by non-allergic rhinitis.

According to the results of our analyses, none of the flavonoids subclasses considered are significantly associated with chronic bronchitis, which reflects what has been previously reported in a case-control study on the general population. [43] We found a statistically non-significant association between anthocyanin intake and a reduced risk of chronic bronchitis. Even though inflammation is an important aspect of chronic bronchitis, the avoidance of the environmental pollutants could be more important to reduce the risk of this disease. [11]

The association between flavonoids and allergic rhinitis has been studied in some observational studies. According to a study conducted by Ross S. M., flavonoids contained in the extract of French maritime pine bark could relieve the symptoms of eyes and nose; the association was not statistically significant, probably because a low number of subjects was included in the study. [76] Enzymatically modified isoquercitrin (EMIQ) was found to prevent ocular symptoms but not nasal symptom, according to another clinical study. [77]

An increased risk of PA was found comparing the 2nd quartile of intake of flavones with the first quartile, and the association was significant in both the unadjusted and adjusted model. This significant association, however, was not consistent with the continuous model and the p-value of the trend was not significant. More-

over, the comparison of the extreme quantiles (4th vs. 1st in this case) is usually more informative when exploring flavonoids intake, as these compounds are widely found in many food sources.

This study presents many advantages. Many chronic respiratory diseases were considered simultaneously, providing a broad view of the effect that flavonoids could have on diverse pathophysiological conditions. Cases and controls were classified in two steps, first by collecting information on symptoms and use of medicines and second by a clinical visit. [46] Moreover, we used a reliable instrument to assess the flavonoid intake; in fact, the validation of the EPIC FFQ was assessed by comparing multiple interviews and through the analysis of nitrogen in urine samples. [53] Furthermore, in order to explore widely the possible effect of different flavonoids, we estimated the 7 main subclasses by using the information retrieved from USDA database, which is an updated source of information on flavonoids content of foods. The most common potential confounders were accurately selected on the basis of the pre-exiting knowledge; [67] further to this, total fruit intake and vitamin C intake were inserted in the model to reduce the possible confounding effect of an healthy diet rich in fruits, and the potential antioxidant effect of Vitamin C.

We are aware that some limitations of the present study should be acknowledged. The statistical power could have been affected by the presence of only one control group and five case groups, which could have reduced the capacity of detect significant differences in the participants. Despite we considered the potential confounders according to the existing knowledge, there is the possibility of the presence of residual confounding, and inserting a healthy dietary score as potential confounder would be advised in future studies to adjust for the overall effect of a healthy or deleterious diet. [78] Among the potential confounders, smoking status was considered, however, according to some studies, it could act as effect modifier. [59] We could estimate the daily intake of the major flavonoid subclasses thanks to the extensive list of vegetable foods provided by the Italian version of the EPIC FFQ. A previous validation study on this questionnaire, however, showed that there is only a moderate level of agreement in the consumption of some foods and some macronutrients, [53] and the EPIC FFQ is not specifically validated for the

estimation of flavonoids intake. Another important aspect that must be considered is that the estimation of nutrients from dietary questionnaires do not take into account the possible interaction among the nutrient and different absorption rate, thus the analysis of nutritional biomarkers would be recommended to further improve the precision of the results and reducing the errors on the nutrient intake estimation. [79] Finally, dietary habits report and clinical assessment of cases and controls were performed in a relatively short distance of time and this could represent a limit for the causal association assessment. An earlier study, however, showed that in adults the dietary habits remain relatively stable from $5$ to $6$ years. [80] Some limitation in the classification of the subjects should be taken into account. As mentioned above, the definition of cases and controls were carefully assessed in two steps. Dichotomic classification of the diseases, however, have raised some concern as this could represent a limitation in epidemiological studies, as some diseases like asthma are more likely a continuum condition rather than dichotomous. [81] Bronchodilator challenge test was performed only on participants with a $\text{FEV}_1/\text{FVC} < 70\%$ or $<$ LLN, however some asthmatic subjects could have a ratio higher than the $80\%$ and, at the same time, an important improvement after the administration of a bronchodilator. [82] This could have caused a reduction of the identification of the asthmatic subjects, and consequently affect the accuracy of the analyses. Subjects were classified as having rhinitis if they reported to have had nasal symptoms, but the diagnosis was not clinically confirmed and SPT, although is an effective tool to check for atopy, needs further clinical investigations to confirm the disease. [83, 84] In this work, multiple comparisons were made with the objective of exploring the potential beneficial effect of all the main categories of flavonoids, but no adjustment for multiple comparisons was performed. According to what Kenneth J. Rothman suggested, [85] the adjustment for multiple comparisons could hide possible new findings, reducing type $1$ error but increasing type $2$ error.

In summary, the findings of the present multi-case-control study provide an evidence that flavanones, a subclass of flavonoids mainly contained in citrus fruits, could protect against the risk of having NAR. The other subclasses of flavonoids analyzed were not significantly associated with the risk of the diseases considered.

# Appendix A

# Other Publications

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ORIGINAL ARTICLE

Asthma and Rhinitis

WILEY

# Dietary fats, olive oil and respiratory diseases in Italian adults: A population-based study

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## Summary

**Background:** Fat intake has been associated with respiratory diseases, with conflicting results.

**Objective:** We studied the association between asthma and rhinitis with dietary fats, and their food sources in an Italian population.

**Methods:** Clinical and nutritional information was collected for 871 subjects (aged 20-84) from the population-based multi-case-control study Genes Environment Interaction in Respiratory Diseases (GEIRD): 145 with current asthma (CA), 77 with past asthma (PA), 305 with rhinitis and 344 controls. Food intake was collected using the EPIC (European Investigation into Cancer and Nutrition) Food Frequency Questionnaire. The associations between fats and respiratory diseases were estimated by multinomial models. Fats and their dietary sources were analysed both as continuous variables and as quartiles.

**Results:** Monounsaturated fatty acids and oleic acid were associated with a reduced risk of CA in both continuous (RRR = 0.68, 95%CI: 0.48; 0.96; RRR = 0.69; 95%CI: 0.49; 0.97, per 10 g, respectively) and per-quartile analyses ($p$ for trend = 0.028 and 0.024, respectively). Olive oil was associated with a decreased risk of CA (RRR = 0.80; 95%CI: 0.65; 0.98 per 10 g). An increased risk of rhinitis was associated with moderate total fat and SFA intake.

**Conclusions:** High dietary intakes of oleic acid and of olive oil are associated with a lower risk of asthma but not of rhinitis.

## 1 | INTRODUCTION

Asthma is one of the most common airway diseases and is associated with bronchial hyperresponsiveness (BHR), inflammation and recurrent airway obstruction.[1] Asthma and other allergic diseases such as rhinitis have a high prevalence in developed countries, affecting between 5% and 40% of the European adult population.[2] The high prevalence in asthma and allergy has been suggested to be related to environment and lifestyle habits, being diet an important factor.[3] Recent studies focused on the components of the Western diet (rich in processed food and with fewer sources of high quality fatty acids such as olive oil) which may contribute to allergic sensitization. A particular consideration was put on dietary fat intake, with overall conflicting results.[3] Notably, former studies were mainly conducted among populations following a typical Western diet.[4] On the other side, few data are available for Mediterranean countries where an high intake of olive oil is traditionally observed.[3]

Olive oil, the principal source of fat in Mediterranean diet,[5] is a rich source of monounsaturated fatty acids (MUFA) and of several bio-compounds including polyphenols. Different studies highlight the protective role of oleic acid (the primary component of olive oil) in cardiovascular diseases and cancers[6,7] while information on the respiratory system is scarce. In animals, some studies have shown that olive oil supplementation reduces airway inflammation and bronchial hyperresponsiveness in experimental models of induced asthma[8] and allergic airways disease.[9] Moreover, a reduced risk of wheeze was seen in offspring of mothers assuming a regular intake of olive oil during pregnancy[10] and epidemiological evidence has shown that in children, regular consumption of olive oil is associated with lower prevalence of doctor-diagnosed asthma.[11] Several studies have concluded that a Mediterranean diet might contribute to reduce the prevalence and severity of asthma, in children[12,13] and in adults,[14] but the specific effect of olive oil on respiratory illness in adults has been seldom investigated in population-based studies. Data regarding rhinitis are even less abundant, in particular when addressing adult populations.[15]

In a population-based multi-case-control study of Italian adults, we investigated the association of dietary fatty acids and of olive oil with asthma and rhinitis, using the data collected in the Gene—Environment Interactions in Respiratory Disease (GEIRD) study.

## 2 | METHODS

### 2.1 | Study design

The GEIRD project is a population-based multi-case-control study involving seven Italian centres (Ancona, Palermo, Pavia, Sassari, Terni, Torino and Verona as the co-ordinating centre).[16] In the first stage of the study, new random samples or pre-existing randomly sampled cohorts[17,18] from the general population (20-84 years of age, male/female = 1/1) were mailed a questionnaire on respiratory symptoms. In the second stage, all the subjects reporting symptoms suggestive of chronic bronchitis (CB), chronic obstructive pulmonary disease (COPD) or asthma, and random samples of subjects reporting symptoms of rhinitis and without symptoms were invited to clinics.

All measurement protocols were in agreement with international guidelines (www.geird.org).[16] Ethical approval was obtained in each centre from the appropriate ethics committee. The ethical committee of the co-ordinating centre is the Comitato Etico per la Sperimentazione - Azienda Ospedaliera di Verona. Written informed consent was obtained from each participant.

For this analysis, only the subjects recruited in the centres of Pavia, Sassari, Torino and Verona were considered. The other centres were excluded because of the lack of complete information on dietary and/or spirometric data.

The number of subjects eligible for the screening phase in these centres was 18 543, out of them 10 873 (58.6%) filled in the postal questionnaire. Overall, out of 6011 subjects invited to clinics, 2189 (36.4%) attended the clinical stage (Figure S1 in the Online Repository). Out of the 2189 subjects participating in the clinical stage, 994 (45%) filled in the FFQ.

### 2.2 | Clinical visit

Each subject underwent a detailed interview. During this interview, the information on gender, birth date and age at completing full-time education was collected, as well as the information on self-reported smoking habits and physical activity (estimated by asking participants how often—frequency—and for how many hours—duration—a week they usually exercised so much that they got out of breath or sweaty).

The subjects were weighed to the nearest 0.1 kg with light clothing and no shoes, and height was measured to the nearest 0.5 cm while they stood barefoot.

### 2.3 | Lung function and skin prick test (SPT) measurements

During the clinical visit, lung function tests and SPT were performed in order to define the case-control status of the subjects. Participants underwent forced spirometry according to the ATS reproducibility criteria.[19] $FEV_1$% predicted (Forced Expiratory Volume in the 1st second) and the lower limit of normal (LLN) for the $FEV_1$/FVC (Forced Vital Capacity) were calculated on the basis of Quanjer et al equation.[20] Subjects with a $FEV_1$/FVC $\geq$70% and $\geq$LLN underwent methacholine challenge test, which followed a protocol described elsewhere.[21] Subjects with a $FEV_1$/FVC <70% or <LLN underwent the bronchodilator challenge test and were invited (if eligible) to undergo the methacholine challenge test. Skin prick tests to 14 common allergens were carried out as described elsewhere[22] and atopy was defined as having a >3 mm reaction.

### 2.4 | Food frequency questionnaire (FFQ)

The subjects also answered a FFQ. The FFQ used is the Italian version of the validated European Investigation into Cancer and

Nutrition (EPIC) questionnaire.[23] The NAF software (Nutritional Analysis of Food Frequency Questionnaires, National Cancer Institute, Milan, Italy)[24] was used to derive daily food intake (grams) and to estimate macro- and micro-nutrient composition. Nutrient data for specific foods consumed in Italy were obtained from the food composition database for epidemiological studies in Italy.[25] Some implausibly high and low intakes of nutrients resulted from the questionnaire, so cut-points were set to exclude outliers. Seventeen subjects with less than 70% of the total number of questions were excluded from the analyses. Then, the ratio of energy intake (EI) to basal metabolic rate (BMR; EI:BMR) was calculated, where BMR was estimated using sex-specific equations for adults (≤60 year old)[26] and for elderly subjects (>60 year old).[27] Cut-points based on the top and bottom 0.5% of the distribution of EI:BMR were introduced, and eight more subjects were excluded. Moreover, we excluded seven subjects with extremely low levels (<600 kcal for women and <800 kcal for men) of EI.[28]

The final number of subjects with clinical and nutritional information was 962.

## 2.5 | Identification of cases and controls in clinics

The 962 subjects were hierarchically classified as follows:

- 145 cases of current asthma, CA:
  - a reported history of asthma *and* asthma-like symptoms/medicines in the last 12 months;
  - a reported history of asthma *or* asthma-like symptoms/medicines in the last 12 months *plus* one of the following conditions: (a) a positive methacholine challenge test with a provocative dose of methacholine causing a 20% drop in $FEV_1$ (PD20) <1 mg; (b) pre-bronchodilator $FEV_1$/FVC <70% or <LLN[19] with a positive reversibility test (i.e. $FEV_1$ >12% and >200 mL after the administration of 400 μg of salbutamol); and (c) pre-bronchodilator $FEV_1$/FVC <70% or <LLN with a post-bronchodilator $FEV_1$/FVC >LLN and >70% and a post-bronchodilator $FEV_1$ >80% predicted[19];
- 77 cases of past asthma, PA: a history of asthma that did not fulfil the criteria for CA;
- six cases of COPD: post-bronchodilator $FEV_1$/FVC <70% or <LLN without asthma;
- 305 cases of rhinitis: reported nasal allergies or nasal symptoms;
- 344 controls: no nasal/respiratory symptoms/conditions reported *plus* both (a) pre-bronchodilator $FEV_1$/FVC > LLN and >70% and (b) $FEV_1$ >70% predicted;
- 85 subjects could not be classified.

The controls were not paired to the cases.

The six subjects with COPD and the 85 unclassified were excluded from the analyses.

## 2.6 | Dietary exposures

The following dietary sources of fats were included as dietary exposures of interest:

1. Macro-nutrients: total fatty acid intake, fractional fatty acid groups namely MUFA, saturated fats (SFA), polyunsaturated fatty acids (PUFA), oleic acid, animal fats and vegetable fats.
2. Olive oil and butter.

The following covariates were considered as potential confounders: study sample/cohort, centre, gender, age, body mass index (BMI, computed dividing weight by height squared), education (low = completed before the age of 16) as a proxy of socio-economic status, smoking habit (never smoker, past smoker i.e. not smoking in the last month, current smoker), self-reported physical activity (heavy, moderate and light), alcohol intake, total protein intake and total EI.

Alcohol intake, total protein intake and total EI were determined based on the information provided in the FFQ.

## 2.7 | Statistical analyses

Our primary exposures of interest (different types of fats, oleic acid, butter and olive oil) were energy-adjusted according to the residual method.[29] According to the residual method, the exposure residuals obtained by regressing the exposure intake on total EI are included as independent variables. Total EI is also included as a covariate. We used log transformation of the dietary intake variables to create residuals with a more constant variance across the levels of total EI.[29] To express nutrients and foods in a more acquainted scale, a back-transformation was then made by adding a constant (the predicted value for the logarithm of the mean total EI) and then taking the antilogarithm.[30]

The main exposures of interest were considered as continuous variables, and they were also categorized into quartiles based on the distribution of the exposure in controls.

To investigate the associations of dietary exposures of interest and case-control status, several multinomial regression models were fitted to the data, using a 4-level dependent variable (CA, PA, rhinitis, and control). Since different types of fats are inter-correlated due to the same food sources, we followed the suggestion by Hu et al,[30] who recommend to adjust fats simultaneously for each other in the analyses. The multinomial regression models were built to include: (a) animal and vegetable fat; (b) SFA, MUFA and PUFA; and (c) oleic acid, SFA and PUFA, in the same model. In addition, we considered two types of food containing fat: olive oil and butter. We assessed the associations between olive oil and case-control status and between butter and case-control status in separate models.

Multivariable associations of exposures with case-control status were expressed by relative risk ratios (RRRs; using control as the reference category) and their 95% CIs. These associations were determined for dietary exposures either as continuous variables or in

quartiles. To test for linear trend across intake quartile categories, we assigned the median intake of each quartile category to everyone with intakes in the category and then we included this quartile median variable as a continuous factor in the statistical models. The *P*-value for trend was the resulting *P*-value for the associated model coefficient.

The statistical analysis was performed using STATA software, release 15.0 (Stata Corp, College Station, TX, USA).

## 3 | RESULTS

### 3.1 | Participation in the nutritional protocol

The distribution of socio-demographic and lifestyle factors was compared among the participants, as opposed to the non-participants in the nutritional protocol, separately in cases and in controls. In each group of cases and in the controls, the subjects who participated in the nutritional protocol were similar to the subjects who did not with regard to the distribution of gender, smoking habits, drinking habits, BMI and education level. Age was significantly associated to participation in the nutritional protocol, in particular in subjects with CA (mean age: 45.6 and 49.5 years, in non-participants and participants, respectively, $P = 0.001$) and with rhinitis (mean age: 47.7 and 50.4 years, in non-participants and participants, respectively, $P = 0.02$), but not in PA, and in controls. Subjects with rhinitis participating in the clinical protocol were significantly more physically active than subjects not participating ($P = 0.02$). (Table S1 in the Online Repository)

### 3.2 | Main characteristics of cases and controls

The distribution of gender, smoking and drinking habits, total daily alcohol intake, BMI and self-reported physical activity was not significantly different between cases and controls (Table 1). Age was significantly different across groups: mean age was comprised between 44.9 years in subjects with PA and 51.5 years in controls.

### 3.3 | Association between fats and respiratory diseases

Table 2 shows the median, 1st and 3rd quartiles of fats and selected food intakes for subjects with and without respiratory diseases.

Intakes of MUFA and oleic acid were associated with a reduced risk of CA. When considering fat intake as a continuous variable, the risk to be a case of CA rather than a control decreased by about 30% for an increase of 10 g/d in the MUFA intake (RRR = 0.68; 95% CI: 0.48; 0.96). A similar decrease was found for oleic acid (RRR =

**TABLE 1** Main characteristics of the subjects participating in the nutritional protocol by case-control status in the GEIRD study

| | Controls (n = 344) | CA (n = 145) | PA (n = 77) | Rhinitis (n = 305) | *P* |
|---|---|---|---|---|---|
| Age at the clinical visit, years (mean, SD) | 51.5 (11.5) | 49.5 (11.7) | 44.9 (11.4) | 50.4 (12.6) | **<0.001** |
| Gender (%) | | | | | |
| Male | 48.0 | 47.6 | 44.2 | 48.9 | 0.91 |
| Smoking habits (%) | | | | | |
| Non-smoker | 52.2 | 49.0 | 56.6 | 50.8 | 0.24 |
| Ex-smoker | 32.9 | 27.6 | 26.3 | 28.2 | |
| Current smoker | 14.9 | 23.4 | 17.1 | 21.0 | |
| Drinking habits (%) | | | | | |
| Current drinker | 35.3 | 46.9 | 36.4 | 41.8 | 0.08 |
| Total alcohol (g/d) | | | | | |
| Abstainers | 64.0 | 52.4 | 63.6 | 56.6 | 0.09 |
| Ex-drinkers | 1.5 | 0.7 | 0.0 | 2.0 | |
| <5 | 7.1 | 15.9 | 10.4 | 9.9 | |
| 5-15 | 17.1 | 17.2 | 18.2 | 14.2 | |
| 15-30 | 6.2 | 8.3 | 5.2 | 11.6 | |
| 30-120 | 4.1 | 5.5 | 2.6 | 5.6 | |
| BMI, kg/m$^2$ (mean, SD) | 25.4 (4.7) | 25.2 (4.2) | 24.8 (4.4) | 25.3 (4.1) | 0.68 |
| BMI, kg/m$^2$ (median, 1st quartile, 3rd quartile) | 25.0 (22.3;27.8) | 24.7 (21.8;27.5) | 24.0 (21.7;26.9) | 24.4 (22.5;27.4) | 0.62 |
| Physical activity (%) | | | | | |
| Heavy | 4.9 | 4.8 | 6.5 | 8.5 | 0.24 |
| Moderate | 37.8 | 35.9 | 31.2 | 40.7 | |
| Light | 57.3 | 59.3 | 62.3 | 50.8 | |
| Education level (%) | | | | | |
| High | 74.1 | 76.6 | 85.7 | 73.9 | 0.16 |

Statistically significant *P*-values are shown in bold.

**TABLE 2** Median, first (p25) and third (p75) quartile of fat intake and of selected foods (g/die) in subjects without respiratory diseases (controls), and in subjects with CA, PA and rhinitis in the GEIRD study

| | Controls (n = 344) | | CA (n = 145) | | PA (n = 77) | | Rhinitis (n = 305) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Median | p25;p75 | Median | p25;p75 | Median | p25;p75 | Median | p25;p75 | *P* |
| Total fat | 72.4 | 56.5;94.1 | 71.9 | 57.4;89.7 | 80.4 | 59.1;107.5 | 77.0 | 60.5;96.1 | 0.07 |
| Animal fat | 39.9 | 26.9;53.5 | 38.4 | 29.4;51.7 | 42.5 | 29.8;58.8 | 42.5 | 30.3;54.7 | 0.41 |
| Vegetable fat | 33.4 | 22.5;42.3 | 32.8 | 21.0;39.0 | 35.3 | 26.2;47.0 | 32.6 | 25.0;42.6 | 0.12 |
| Saturated fat | 24.9 | 18.6;32.7 | 24.0 | 19.0;32.2 | 24.8 | 20.6;37.3 | 26.9 | 19.4;33.2 | 0.20 |
| Monounsaturated fat | 35.4 | 27.1;44.7 | 35.2 | 25.9;41.7 | 37.0 | 29.4;49.8 | 36.6 | 28.7;46.0 | 0.07 |
| Polyunsaturated fat | 8.2 | 6.5;10.7 | 8.0 | 6.2;10.3 | 9.2 | 6.9;11.5 | 8.5 | 6.8;10.7 | 0.07 |
| Oleic acid | 33.5 | 25.4;42.1 | 32.9 | 24.1;38.9 | 35.3 | 27.7;48.0 | 34.1 | 26.8;43.2 | 0.08 |
| Olive oil | 21.2 | 14.1;29.4 | 19.8 | 12.9;27.2 | 22.2 | 18.1;33.9 | 21.1 | 15.0;30.1 | 0.06 |
| Butter | 0.3 | 0.1;0.9 | 0.4 | 0.1;1.1 | 0.3 | 0.1;0.4 | 0.4 | 0.1;1.0 | 0.38 |

0.69; 95%CI: 0.49; 0.97) (Table 3). A reduced risk of CA was detectable even when MUFA and oleic acid were categorized in quartiles. For both kinds of fat, the risk of having CA decreased as fat intake increased with a significant *P*-value for trend (*P* = 0.03 and 0.02, for MUFA and oleic acid, respectively), and the subjects in the highest quartile of MUFA and oleic acid intake had less than half the risk of having CA with respect to the subjects in the lowest quartile (RRR = 0.44; 95%CI: 0.21; 0.95 and RRR = 0.42; 95%CI: 0.20; 0.88, for MUFA and oleic acid, respectively; Table 4).

The risk to be a subject with CA, rather than a control, decreased when the intake of vegetable fat increased, though not significantly (*P*-value for trend = 0.06). The RRR for the highest vs lowest quartile was 0.49 (95%CI: 0.26; 0.93; Table 4).

An increase in the risk of rhinitis occurred only for a modest increase in the SFA intake and in the total fat intake (i.e. in the 2nd quartile of intake vs 1st; Table 4). However, there was no increase in trend of rhinitis by fat intake (Table 4).

## 3.4 | Association between selected foods and respiratory diseases

Olive oil was associated with a reduced risk of CA; in particular, when considering olive oil intake as a continuous variable, the risk to be a case of CA, rather than a control, decreased by 20% for an increase of 10 g/d in olive oil intake (RRR = 0.80; 95%CI: 0.65; 0.98) (Table 5). A consistent trend was evident when observing the RRRs of CA for quartiles of olive oil intake, which decreased from 0.88 for the 2nd quartile to 0.58 for the 4th quartile; however, this trend was only borderline significant (*P* for trend = 0.06) (Table 6). An increased risk of PA was associated with a high intake of olive oil (4th quartile vs

**TABLE 3** Adjusted RRR (and 95%CI) to be a case of CA, PA and rhinitis, rather than a control (n = 344), according to the intake of fats in the GEIRD study

| | CA (n = 145) | PA (n = 77) | Rhinitis (n = 305) |
|---|---|---|---|
| Animal fat (10 g) | 0.90 (0.72;1.13) | 1.01 (0.74;1.37) | 1.10 (0.92;1.32) |
| Vegetable fat (10 g) | 0.81 (0.66;1.01) | 1.24 (0.96;1.62) | 1.07 (0.91;1.26) |
| Total Energy (100 kcal) | 0.98 (0.95;1.01) | 1.03 (0.99;1.07) | 1.00 (0.98;1.03) |
| Saturated fat (10 g)[a] | 1.02 (0.68;1.55) | 0.91 (0.53;1.57) | 1.13 (0.82;1.57) |
| Monounsaturated fat (10 g) | 0.68 (0.48;0.96)* | 1.26 (0.82;1.93) | 1.05 (0.80;1.37) |
| Polyunsaturated fat (1 g)[a] | 1.04 (0.93;1.17) | 1.04 (0.89;1.23) | 1.01 (0.91;1.11) |
| Total Energy (100 kcal) | 0.98 (0.95;1.01) | 1.03 (0.99;1.07) | 1.00 (0.98;1.03) |
| Saturated fat[a] | 0.99 (0.66;1.49) | 0.93 (0.54;1.58) | 1.14 (0.82;1.57) |
| Oleic acid (10 g) | 0.69 (0.49;0.97)* | 1.26 (0.82;1.91) | 1.04 (0.80;1.36) |
| Polyunsaturated fat (1 g)[a] | 1.04 (0.93;1.16) | 1.05 (0.89;1.23) | 1.01 (0.92;1.11) |
| Total Energy (100 kcal) | 0.98 (0.95;1.01) | 1.03 (0.99;1.07) | 1.00 (0.98;1.03) |
| Total fat (10 g) | 0.85 (0.71;1.02) | 1.15 (0.91;1.45) | 1.08 (0.93;1.24) |
| Total Energy (100 kcal) | 0.98 (0.95;1.02) | 1.03 (0.99;1.07) | 1.00 (0.98;1.03) |

The estimates were adjusted for age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, physical activity, educational level, total protein intake and total energy intake. The marked areas in grey/white separate the variables comprised in different models.

[a]Two different, but generally similar, RRRs are proposed for the association of SFA with each considered disease, due to the fact that SFA was included in two different models: one model with MUFA as a covariate and the other one with oleic acid as a covariate. The same applies for PUFA.

*$P < 0.05$.

**TABLE 4** Adjusted RRR (and 95%CI) to be a case of CA, PA and rhinitis, rather than a control (n = 344), according to quartiles of fat intake (based on the distribution of controls) in the GEIRD study

| | Quartile of intake | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | *P* (Trend) |
| CA (n = 145) | | | | | |
| Animal fat | 1.00 | 1.30 (0.72;2.35) | 0.88 (0.46;1.69) | 1.09 (0.55;2.14) | 0.90 |
| Vegetable fat | 1.00 | 0.77 (0.44;1.54) | 0.87 (0.49;1.54) | 0.49 (0.26;0.93)* | 0.06 |
| Saturated fat[a] | 1.00 | 1.43 (0.79;2.61) | 1.05 (0.55;2.00) | 1.40 (0.71;2.76) | 0.57 |
| Monounsaturated fat | 1.00 | 0.80 (0.44;1.44) | 0.58 (0.30;1.11) | 0.44 (0.21;0.95)* | **0.03** |
| Polyunsaturated fat[a] | 1.00 | 0.91 (0.50;1.65) | 1.03 (0.64;1.97) | 1.09 (0.53;2.21) | 0.78 |
| Saturated fat[a] | 1.00 | 1.45 (0.79;2.64) | 1.05 (0.55;1.99) | 1.37 (0.70;2.69) | 0.61 |
| Oleic acid | 1.00 | 0.67 (0.37;1.22) | 0.59 (0.31;1.12) | 0.42 (0.20;0.88)* | **0.02** |
| Polyunsaturated fat[a] | 1.00 | 0.94 (0.52;1.70) | 1.04 (0.54;1.97) | 1.09 (0.54;2.21) | 0.78 |
| Total fat | 1.00 | 0.98 (0.56;1.69) | 0.58 (0.31;1.08) | 0.62 (0.33;1.16) | 0.06 |
| PA (n = 77) | | | | | |
| Animal fat | 1.00 | 2.02 (0.94;4.37) | 0.91 (0.37;2.20) | 1.24 (0.49;3.14) | 0.80 |
| Vegetable fat | 1.00 | 1.04 (0.49;2.61) | 1.13 (0.49;2.61) | 1.78 (0.79;4.00) | 0.14 |
| Saturated fat[a] | 1.00 | 1.12 (0.52;2.41) | 0.59 (0.25;1.37) | 0.74 (0.31;1.81) | 0.28 |
| Monounsaturated fat | 1.00 | 1.30 (0.57;2.96) | 0.87 (0.35;2.19) | 1.60 (0.57;2.96) | 0.49 |
| Polyunsaturated fat[a] | 1.00 | 1.50 (0.63;3.58) | 1.87 (0.77;4.57) | 1.39 (0.52;3.58) | 0.53 |
| Saturated fat[a] | 1.00 | 1.15 (0.53;2.47) | 0.61 (0.26;1.43) | 0.78 (0.32;1.87) | 0.31 |
| Oleic Acid | 1.00 | 1.25 (0.56;2.78) | 0.66 (0.26;1.67) | 1.50 (0.61;3.71) | 0.59 |
| Polyunsaturated fat[a] | 1.00 | 1.51 (0.63;3.58) | 1.97 (0.82;4.76) | 1.46 (0.57;3.85) | 0.44 |
| Total fat | 1.00 | 2.18 (1.01;4.71)* | 1.01 (0.42;2.43) | 1.48 (0.63;3.49) | 0.86 |
| Rhinitis (n = 305) | | | | | |
| Animal fat | 1.00 | 1.61 (1.01;2.58) | 1.06 (0.63;1.78) | 1.46 (0.85;2.52) | 0.43 |
| Vegetable fat | 1.00 | 0.71 (0.44;1.15) | 1.06 (0.66;1.69) | 0.96 (0.59;1.57) | 0.72 |
| Saturated fat[a] | 1.00 | 1.85 (1.13;3.03)* | 1.38 (0.82;2.32) | 1.72 (0.99;2.99) | 0.21 |
| Monounsaturated fat | 1.00 | 0.99 (0.60;1.62) | 0.74 (0.43;1.27) | 0.90 (0.50;1.63) | 0.54 |
| Polyunsaturated fat[a] | 1.00 | 0.78 (0.48;1.28) | 1.13 (0.67;1.89) | 0.89 (0.50;1.57) | 0.99 |
| Saturated fat[a] | 1.00 | 1.82 (1.11;2.97)* | 1.35 (0.81;2.27) | 1.66 (0.96;2.87) | 0.25 |
| Oleic Acid | 1.00 | 1.01 (0.62;1.64) | 0.82 (0.49;1.40) | 0.95 (0.54;1.70) | 0.72 |
| Polyunsaturated fat[a] | 1.00 | 0.76 (0.47;1.25) | 1.09 (0.65;1.81) | 0.87 (0.49;1.52) | 0.91 |
| Total fat | 1.00 | 1.60 (1.00;2.56)* | 1.28 (0.78;2.11) | 1.38 (0.83;2.32) | 0.43 |

Statistically significant *P*-values for trend are shown in bold.
The estimates were adjusted for age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, physical activity, educational level, total protein intake and total energy intake. The marked areas in grey/white separate the variables comprised in different models.
[a]Two different, but generally similar, RRRs are proposed for the association of SFA with each considered disease, due to the fact that SFA was included in two different models: one model with MUFA as a covariate and the other one with oleic acid as a covariate. The same applies for PUFA.
*$P < 0.05$.

1st: RRR = 2.07; 95%CI: 1.01; 4.26; Table 6). There was no evidence of an association between butter and the considered diseases.

## 4 | DISCUSSION

We investigated the relationship between dietary intake of fatty acids, including their dietary food sources, and the risk of respiratory diseases, within the frame of the GEIRD project.

For the first time, we found a significant inverse association between the dietary intake of MUFA and oleic acid with the risk of CA in a sample of adults from the general population. Of interest, a similar association was found between the consumption of olive oil and CA, whereas the opposite association was identified with PA. The reduced risk of CA with the intake of oleic acid is in contrast with the data reported by Nagel et al who found a significant positive association between dietary oleic acid (lipid numbers C18:1) and margarine with the risk of asthma in adulthood. The authors hypothesize that this association may be explained by an increased intake of *trans*-C18:1, which is high in margarine.[31] In our study, the fact that the main source of oleic acid is olive oil, rich of the *cis* isomer, may justify the apparently opposite findings. In other words, we speculate

**TABLE 5** Adjusted RRR (and 95%CI) to be a case of CA, PA and rhinitis, rather than a control (n = 344), according to the intake of selected foods (olive oil and butter, considered in two separate models) in the GEIRD study

| | CA (n = 145) | PA (n = 77) | Rhinitis (n = 305) |
|---|---|---|---|
| Olive oil[a] (10 g) | 0.80 (0.65;0.98)* | 1.23 (0.97;1.56) | 1.02 (0.87;1.18) |
| Total Energy[a] (100 kcal) | 0.98 (0.95;1.01) | 1.03 (0.99;1.07) | 1.00 (0.98;1.03) |
| Butter (1 g)[b] | 0.99 (0.92;1.06) | 0.95 (0.83;1.08) | 0.98 (0.92;1.04) |
| Total Energy[b] (100 kcal) | 0.99 (0.96;1.03) | 1.04 (1.00;1.09) | 1.00 (0.98;1.03) |

The estimates were adjusted for age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, physical activity, educational level, total protein intake and total energy intake.
[a]The estimates were also adjusted for saturated fat.
[b]The estimates were also adjusted for total fat.
*$P < 0.05$.

that the different isoforms of oleic acid contained in olive oil and margarine may influence the occurrence of respiratory diseases in different ways. Our data indirectly contrast with the findings by Heinrich et al who reported that the energy-adjusted dietary intake of MUFA was positively related to the prevalence of atopy.[32] The different designs and outcomes of the studies (allergic sensitization vs a clinical condition like rhinitis in the present study), the different populations (mainly from Central-Northern Europe in Heinrich's study) and the probable different dietary sources of MUFA (animal derived products in the study by Heinrich) can explain the discordant results.

Consistent with our study are the data reported in the Nurses' Health Study, which found that energy-adjusted intake of MUFA was inversely associated with asthma[33]; the same inverse association between MUFA intake and asthma was shown by Huang et al,[34] although in a cohort of teenagers from Taiwan. The mechanisms at the base of the decreased risk of CA associated with MUFA intake are not clear. MUFA may have an anti-inflammatory effect, as demonstrated in a controlled trial[35] where subjects consuming olive oil (containing a high percentage of MUFA) for 2 months showed a decreased expression of adhesion molecules in peripheral blood mononuclear cells.

To the best of our knowledge, this is the first study showing a reduced risk of CA associated with the intake of olive oil in adults. This indirectly supports a Spanish study where olive oil consumption during pregnancy was found to prevent wheezing in the first year of life of offspring,[10] while a Swedish study found a negative association between olive oil and doctor-diagnosed asthma in children. Of interest, no association between olive oil and CA was found in the latter study.[11]

There is epidemiological evidence that Mediterranean diet is associated with lower asthma prevalence.[12] However, previous investigations did not take olive oil as a specific component into account,[12,36] so that the relationship between this nutrient and asthma has not been studied in detail.

The main active components of olive oil are oleic acid, phenolic derivatives and squalene, which have been found to have antioxidant and anti-inflammatory activity.[37] Since inflammation and oxidative stress are key components in asthma pathogenesis,[38] we have good reason to believe that the properties of olive oil may positively influence the disease development.

Two studies carried out in Denmark[39,40] investigated the effect of fish oil supplementation during pregnancy on the occurrence of asthma in offspring using olive oil as placebo, assuming that the intake of olive oil in the doses provided was inert.[39] Our results are in contrast with the assumption that olive oil is inert in relation to asthma, even if the considered quantities in our study were 5-10 times higher.

The influence of fatty acid consumption on the prevalence of rhinitis and atopic diseases has been object of interest in recent

**TABLE 6** Adjusted RRR (and 95%CI) to be a case of CA, PA and rhinitis, rather than a control (n = 344), according to quartile intake of selected foods (olive oil, butter) (based on the distribution of controls) in the GEIRD study

| | Quartile of intake | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | *P* (Trend) |
| CA (n = 145) | | | | | |
| Olive oil[a] | 1.00 | 0.88 (0.51;1.54) | 0.73 (0.42;1.29) | 0.58 (0.32;1.04) | 0.06 |
| Butter[b] | 1.00 | 1.21 (0.64;2.29) | 1.00 (0.52;1.92) | 1.32 (0.70;2.49) | 0.53 |
| PA (n = 77) | | | | | |
| Olive oil[a] | 1.00 | 1.17 (0.54;2.53) | 0.84 (0.37;1.93) | 2.07 (1.01;4.26)* | 0.07 |
| Butter[b] | 1.00 | 1.04 (0.48;2.26) | 0.63 (0.27;1.48) | 0.60 (0.26;1.41) | 0.15 |
| Rhinitis (n = 305) | | | | | |
| Olive oil[a] | 1.00 | 1.03 (0.65;1.62) | 0.71 (0.44;1.14) | 0.91 (0.58;1.45) | 0.40 |
| Butter[b] | 1.00 | 0.87 (0.52;1.44) | 0.93 (0.56;1.54) | 1.00 (0.61;1.64) | 0.94 |

The estimates were adjusted for age, gender, centre, study cohort, BMI, smoking habits, alcohol intake, physical activity, educational level, total protein intake and total energy intake.
[a]The estimates were also adjusted for saturated fat.
[b]The estimates were also adjusted for total fat.
*$P < 0.05$.

years. Some epidemiological studies support the hypothesis that dietary fat intake might play a role in atopy and related diseases.[41,42]

Our study adds a further piece of evidence on the possible role that animal and SFA intake could have in rhinitis.[43] However, our results have to be interpreted with great caution owing to the fact that the increase in the risk of rhinitis occurred only for moderate but not high consumption of animal fat and SFA.

The study strengths are (a) the accurate identification of cases and controls, based either on an extensive clinical interview or on objective clinical tests, (b) the simultaneous comparison of cases of several diseases to controls, (c) the selection of subjects from the general population and (d) the careful dietary assessment using validated food frequency questionnaires.[23,24]

Some caveats should also be taken into account. The rate of participation in the clinical stage was 36%. This could have led to a selection bias; that is, cases and controls consuming a high quantity of lipids were less (or more) prone to participate, even though the scenario seems to be unlikely. Also the participation rate to the FFQ was fairly low (45%), but there were only minor differences between the two groups (participants vs non-participants in the nutritional protocol) related to age, with a very limited, if any, clinical relevance.

There is potential measurement error in the ascertainment of diet.[44] As this is a case-control study, recall bias might have affected the results. No dietary recall was administered to participants; however, the EPIC questionnaire was previously validated in an Italian population sample.[23] Moreover, subjects with a low-quality questionnaire were excluded (see Section 2) and the FFQ provided visual aids for the assessment of portion sizes, likely improving the accuracy of the reported information.

The GEIRD study is a case-control study, and we acknowledge that the relatively short distance between the reporting of dietary habits and case-control definition could constitute a limit for the assessment of a causal association. However, it is of importance to remark that there is evidence that adults maintain relatively stable long-term dietary habits.[45]

## 5 | CONCLUSIONS

The results of this population-based study provide evidence that dietary fats affect the risk of asthma in adults. A high dietary intake of oleic acid and a high consumption level of olive oil were found to decrease the risk of asthma. These results suggest that a diet rich in olive oil, which plays a central role in the Mediterranean diet, besides having beneficial effects against cardiovascular diseases, may also be useful for the respiratory system.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTION

LCazzoletti and MF conceived and designed the study; LCazzoletti, MEZ, RB, IC, AG, PP and MF contributed to the data collection; LCazzoletti performed the statistical analysis; LCazzoletti, MF, MEZ and FS drafted the manuscript; and LCazzoletti, MEZ, FS, RB, LChamitava, IC, VGL, AG, VM, PP and MF contributed to the interpretation of data, revised the paper critically for important intellectual content and approved the version to be published.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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food & nutrition research

ORIGINAL ARTICLE

# Vegetable but not animal protein intake is associated to a better physical performance: a study on a general population sample of adults

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**Popular scientific summary**

- Few studies have been conducted on the separate effect of animal and vegetable protein on physical performance.
- Analysing this relationship, we found an unexpected result, suggesting that a higher vegetable protein intake is associated with a better performance at 6-min walking test.
- Whether this result is related to the high protein intake itself or may be a consequence of the other properties of plant-based foods deserves further investigation.

*Abstract*

***Background:*** The research was conducted in the frame of a population-based, case control study, called Genes Environment Interaction in Respiratory Disease.
***Objective:*** To assess the association between protein intake and physical performance in a general population sample.
***Design:*** Researchers investigated the association between the participants' dietary information and their physical performance using the 6-min walking test and the distance walked in metres (6MWD) as main outcome measure. Information on dietary intake was collected using the validated European Investigation into Cancer and Nutrition food frequency questionnaires (FFQs). Then, daily intake of energy and macronutrients was estimated by means of the NAF software (nutritional analysis of FFQ). Linear regression models were used to evaluate the associations between vegetable, animal and total protein intakes and the 6MWD. The models were adjusted for socio-demographic features, total fats and available carbohydrate intakes.
***Results:*** The participants were 223 subjects (57% females) aged between 23 and 68 years. Their mean vegetable and animal proteins intake for gram/kg of body weight/day were, respectively, 0.4 and 0.7. After adjusting for all the potential confounders, there was a significant increase of 20.0 (95% CI 0.8; 39.2) m in the distance walked for an increase in 10 g/day of vegetable proteins and non-significant variations of −1.8 (95% CI −9.3; 5.7) m for an increase in 10 g/day of animal proteins and of 0.5 (95% CI −6.8; 7.7) for an increase in 10 g/day of total proteins.
***Discussion and conclusions:*** Our result suggests a positive role of vegetable proteins on physical performance. Whether this result is related to the high protein intake itself or may be a consequence of the other properties of plant-based foods deserves further investigation.

Keywords: *six minute walking test; nutrition; diet; exercise; proteins*

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A poor physical performance tested by objective measures of physical capacity seems to play a significant role in predicting an increased risk of mortality and morbidity in the general elderly adult population (1–3). In the last decades, the accumulation of evidence has provided new findings on the impact of





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the nutritional status on the health and functional capacity in the general population (4, 5). It is well known that an adequate intake of quality protein is a key factor for building, preserving muscle mass and maintaining physical functions. Currently, the recommended protein intake is 0.8 g/kg body weight/day (bw/day) for adults (6), even though this amount is a rough estimate, based on the minimal protein intake necessary to maintain the nitrogen balance in adults. It has recently been proposed that the protein intake of 1.0–1.2 g/kg bw/day is likely to be the amount that is required to ameliorate muscle health without damaging the renal function (4, 7). As regards the quality of proteins, it is a common opinion to favour those of animal origin since a greater proportion of daily protein intake derived from animal- versus plant-based sources seems to be associated with better muscle maintenance in older adults (8). Furthermore, animal proteins are more easily available and have a higher level of essential amino acids, which increases protein synthesis and anabolism (9, 10). Several previous investigations (11–15) have been conducted on the association between dietary protein intake and physical performance. Data from these studies generally support the effect of animal protein on preserving muscle mass and improving muscle strength in older adults (9, 16, 17). On the contrary, few studies have examined the association of a dietary protein intake, in terms of both quantity and quality, with physical performance measures in middle-aged adults (i.e. aged between 40 and 65 years). In view of the scarcity of evidence on this topic, the present study aimed at investigating the possible relationship between total animal and vegetable protein intake and the distance walked in 6 min in a cohort of subjects from the general population.

## Methods

### *Study design*

The Genes Environment Interaction in Respiratory Diseases (GEIRD) project is a multi-case-control study on respiratory diseases, carried out between 2007 and 2010 in Italy. The sample was randomly selected from the general population in six centres (Pavia, Sassari, Turin, Ancona, Terni and Verona) by using the local health authority records. The GEIRD project's design is described in detail elsewhere (18). In brief, cases of chronic bronchitis, chronic obstructive pulmonary disease, asthma or rhinitis and controls without respiratory symptoms were identified through a two-step design (postal screening, clinical interview). During the clinical interview, subjects performed the 6MWT and filled in a food frequency questionnaire (FFQ). In the present analysis, only subjects without respiratory symptoms or diseases who participated in the study in Verona, with valid information on their usual dietary intake and on the execution of the 6MWT, were considered ($n$ = 223). Written informed consent was obtained from all participants.

### *Dietary information*

Information on the subjects' usual dietary intake was collected by using the Italian version of the validated European Investigation into Cancer and Nutrition (EPIC) FFQ (19). To ensure the quality of participants' dietary reporting, participants who filled less than 70% of the total number of questions , as well as the subjects with an extremely low caloric intake level and those on top and bottom 0.5% of the distribution of Energy Intake/Basal Metabolism Ratio (EI/BMR), were excluded from the study. Then, daily intake of energy and macronutrients was estimated by means of the NAF software (nutritional analysis of FFQs, National Cancer Institute, Milan, Italy) (20), and the information on nutrients for specific foods consumed in Italy was obtained from the food composition database for epidemiological studies in Italy (21).

### *6MWT*

The 6MWT was performed following the American Thoracic Society guidelines (22). Before performing the test, subjects were checked for contraindications. Subjects were asked to walk as fast as they could without running in a 25-m-long hallway, for 6 min. The test results were expressed as the distance walked (6MWD) in metres. Out of 255 controls with available information on their nutritional status, 32 (12.5%) did not perform the 6-min walking test (6-MWT), because of clinical contraindications, including heart attack occurred in the previous 3 months, current drug treatment for epilepsy, a heart rate more than 120 beats per minute and a systolic blood pressure over 180 mmHg or a diastolic blood pressure over 100 mmHg.

### *Exposures and potential confounders*

Vegetable and animal protein intakes (g/day) were considered as determinants. The following covariates were considered as potential confounders: gender, age, height, weight, smoking habits (never smoker, past smoker, i.e. not smoking in the last month or current smoker), comorbidity, self-reported intensity of physical exercise, total fats and available carbohydrates (i.e. the sum of monosaccharides, disaccharides, dextrins, starch and glycogen expressed in monosaccharides). The distribution of the total energy intake in kJ/day (or in kcal/day) was also calculated. According to the participants' questionnaire answers, the comorbidity status was defined by the presence of self-reported medical diagnosis of at least one of the following diseases: arterial hypertension, diabetes, cardiovascular comorbidity (at least one of lifetime heart attack, ictus, angina pectoris, arrhythmia, heart or aorta surgery) and cancer. Intensity of exercise performed during a week



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was classified into three levels and estimated by asking participants how often, and for how many hours weekly they were exercising so as to have a feeling of shortness of breath and to sweat.

### *Statistics*

Subject characteristics were summarised as percentages or means (SD). A two sample *t*-test on the equality of means was performed to investigate the difference in physical performance (6MWD) between subjects ingesting more or less than 0.8 g protein per kg of body mass-daily, which is the recommended daily allowance (RDA) for proteins (23). Models considering the 6MWD as the dependent variable were fitted using a simple linear regression for the nutrient intakes of interest. Then, multiple linear regression models were fitted to the data, with each nutrient intake as the independent variable, adjusting for a first set of potential confounders (gender, age, height, weight, smoking habits, comorbidities and the self-reported intensity of physical training). Another model was fitted considering, in addition to the first set of confounders, also animal proteins, vegetables proteins, total fats and available carbohydrates. Lastly, a multiple regression model similar to the previous one was fitted taking into account the total proteins (expressed as g/day) instead of vegetable and animal proteins separately. All statistical analyses were performed using the software Stata, version 13.0 (www.stata.com).

*Table 1.* Socio-demographic characteristics of participants ($N = 223$)[§]

| | Participants ($n$ = 223)* |
|---|---|
| Age at the clinical visit, years | 45.8; 9.6 |
| Males | 43.0 (96) |
| Females | 57.0 (127) |
| BMI, kg/m$^2$ | 24.4; 4.0 |
| Weight, kg | 70.6; 14.8 |
| Height, cm | 169.6; 9.4 |
| Non smoker | 58.1 (129) |
| Ex-smoker | 28.8 (64) |
| Current smoker | 13.1 (29) |
| Heavy physical activity | 7.6 (17) |
| Moderate physical activity | 44.0 (98) |
| Light physical activity | 48.4 (108) |
| Presence of comorbidity | 24.8 (55) |
| Absence of comorbidity | 75.2 (167) |

[§]Subject characteristics were summarised as percentages (number of observations) or means (SD) related to qualitative and quantitative variables, respectively. *There were missing values on smoking habits (one missing value) and comorbidity (one missing value), so the percentages were calculated considering the total number of subjects after excluding the missing data.

## Results

The socio-demographic characteristics of the 223 participants are shown in Table 1. Their mean age at the clinical visit was 45.8 years (SD = 9.6 years; range = 22.8–68.4 years). Most participants were women (57.0%), had a normal mean value of BMI (24.4 kg; range = 16.9–39.8 kg/m), were non-smoker (58.1%) and reported a light intensity of physical training (48.4%) and the absence of comotbidities (75.2%). The mean value of weight and height were 70.6 (range = 41–110) kg and 169.6 (range = 147–192) cm, respectively (Table 1). Their dietary intake is shown in Table 2. The median total energy intake was 7,924.5 kJ/day (= 1,894 kcal/day). The subjects reported daily median intakes of 48.2 g/day for animal proteins (i.e. 0.7 g/kg bw/day) and 24.3 g/day for

*Table 2.* Nutritional intake estimates of participants ($N = 223$)

| Nutritional dietary intake | Median | First quartile; third quartile | Range |
|---|---|---|---|
| Vegetable protein – g/day | 24.3 | 18.3; 31.2 | 8.2–95.7 |
| Animal protein – g/day | 48.2 | 38.1; 63.7 | 9.5–122.5 |
| Total protein – g/day | 73.3 | 58.5; 93.6 | 22.7–180.6 |
| Vegetable protein–g/kg/day | 0.4 | 0.3; 0.5 | 0.1–1.3 |
| Animal protein–g/kg/day | 0.7 | 0.5; 1.0 | 0.1–2.2 |
| Total proteins–g/kg/day | 1.1 | 0.8; 1.4 | 0.3–2.7 |
| Available carbohydrates – g/day | 235.6 | 174.4; 308.7 | 73.1–748.9 |
| Total fats –g/day | 74.8 | 60.6; 95.6 | 20.6–169.8 |
| Total energy intake – kJ/day<br>[Total energy intake – kcal/day] | 7,926.2<br>[1,894.4] | 62,960.8; 103,989.1<br>[1,504.8; 2,485.4] | 2,985.3–19,491.2<br>[713.5–4,658.5] |
| Total proteins– energy % | 15.5 | 14.1; 17.3 | 9.9–26.3 |
| Vegetable protein–energy % | 5.1 | 4.5; 5.8 | 3.0–8.6 |
| Animal protein–energy % | 10.0 | 8.5; 12.3 | 3.9–22.7 |
| Available carbohydrates – energy% | 49.1 | 43.2; 54.3 | 28.4–71.0 |
| Total fats–energy % | 35.3 | 31.5; 38.9 | 22.5–53.0 |

*Table 3.* Simple and multiple linear regression coefficients (with 95% CIS) for regression of 6MWD (M) against nutrient intakes for an increase of 10 g/day

| Nutritional dietary intake −10 g/day | *b*-Coefficient (simple linear regression) | Adjusted b-coefficient (*) (multiple linear regression) | Adjusted b-coefficient (**) (multiple linear regression) |
|---|---|---|---|
| Animal protein | −0.3 (−4.7; 4.0) | −1.4 (−5.5; 2.8) | −1.8 (−9.3; 5.7) |
| Vegetable protein | **13.2 (5.4; 21.0)** | **9.5 (1.7; 17.3)** | **20.0 (0.8; 39.2)** |
| Total fats | 1.5 (−1.9; 4.9) | 0.1 (−3.2; 3.3) | −1.4 (−8.4; 5.6) |
| Available carbohydrates | **1.3 (0.4; 2.3)** | 0.8 (−0.1; 1.7) | −0.9 (−3.3; 1.5) |

**b*-Coefficient is adjusted for the following covariates: gender, age, height, weight, smoking habits, comorbidities and the self-reported intensity of physical training; ***b*-Coefficient is adjusted for the following covariates: gender, age, height, weight, smoking habits, comorbidities, the self-reported intensity of physical training, vegetable protein (g/day), animal protein (g/day), available carbohydrates (g/day) and total fats (g/day). Regression coefficients that are significantly different from 0 are reported in bold.

vegetable proteins (i.e. 0.4 g/kg bw/day). Table 2 reports daily dietary information reported as percentage of total energy intake.

The majority of the participants (78.9%) reported a protein intake that was more than 0.8 g protein per kg of body mass-daily. The median 6MWD value was 599.4 m (SD = 70.0). There was not a significant difference in the 6MWD between subjects ingesting more or less than 0.8 g protein per kg of body mass-daily (599.8 and 598.1 m, respectively, $P = 0.89$). When considering the simple linear regression, there was a significant increase of 13.2 (95% CI: 5.4; 21.0) m ($P < 0.001$) for 10 g/day increase in the vegetable protein intake (Table 3), and no apparent variation of the 6MWD (−0.3; 95% CI: −4.7; 4.0 m, $P = 0.881$) for 10 g/day increase in the animal protein intake (Table 3). When adjusting for the first set of confounders (gender, age, height, weight, smoking habits, comorbidities and intensity of physical training) and when also adjusting for all the other nutrients (i.e. animal proteins, total fats and available carbohydrates), the positive association between the vegetable protein intake and the 6MWD was confirmed. When considering the latter multivariable regression model, the predicted increase in the 6MWD was 20.0 (95% CI: 0.8; 39.2; $P = 0.041$) m for 10 g/day increase in the vegetable protein intake (Table 3, Fig. 1, upper panel). Total fats (*b* coefficient for 10 g intake increase = −1.4; $P = 0.69$) as well as available carbohydrates (*b* coefficient for 10 g intake increase = −0.9; $P = 0.44$) and animal proteins (*b* coefficient for 10 g intake increase = −1.8; $P = 0.64$) were not associated with the 6MWD (Table 3, last column; Fig. 1, lower panel). Lastly, another multiple regression model was fitted to the data, taking into account the total proteins, instead of vegetable and animal proteins separately, and adjusting for all the above-mentioned confounders, including total fats and available carbohydrates. This model showed that the total protein intake (*b* coefficient for 10 g intake increase = 0.5; 95% CI −6.8; 7.7) was not associated with the 6MWD.

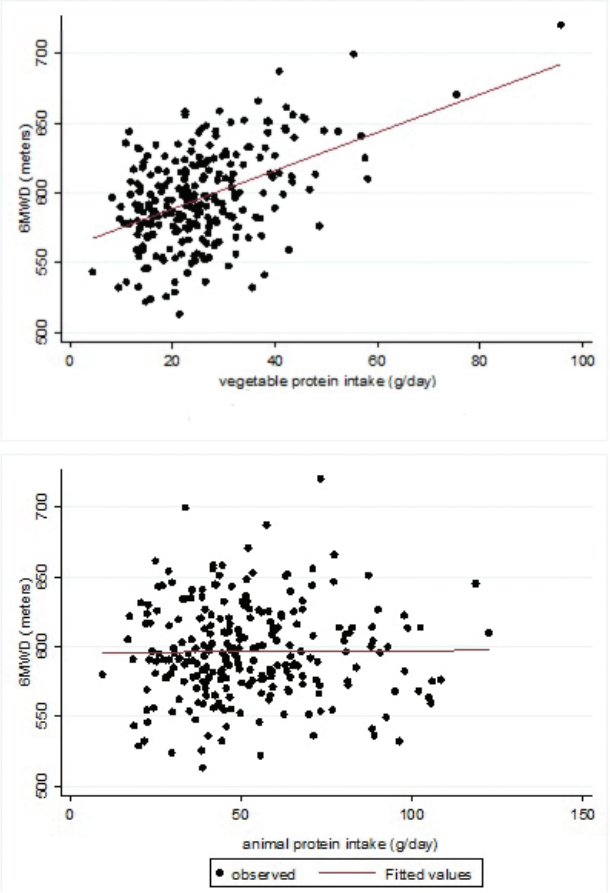


*Fig. 1.* Vegetable protein intake (g/day) or animal protein intake (g/day) and 6MWD (metres). The lines represent the fitted value of the multiple linear regressions where the dependent variable is 6MWD and the independent variable is the vegetable protein intake (upper panel) and the animal protein intake (lower panel). The two models are adjusted for the following covariates: gender, age, height, weight, smoking habits, comorbidities, the intensity of physical training, vegetable protein (g/day), animal protein (g/day), available carbohydrates (g/day) and total fats (g/day).



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## Discussion

Our main result was the finding of a direct relationship between the vegetable protein intake and the distance walked in the 6MWT. It is noteworthy to mention that the association persisted after adjusting for several possible confounders. On the contrary, no significant association was found for animal and total protein intakes. Several previous studies (11–14) have been conducted on the association between dietary protein intake and physical performance. Isanejad et al. (13), studying women aged 65–71 years belonging to the Osteoporosis Risk Factor and Prevention Fracture Prevention Study, demonstrated that subjects with a higher protein intake (≥1.2 g/kg) had a significantly better physical function and muscle strength compared with those with moderate and lower intakes. Gregorio et al. (11) demonstrated that upper and lower extremity function was impaired in older women consuming a low protein diet (below the RDA for protein defined as less than 0.8 g protein/kg) compared to those with a higher protein intake, and Radavelli-Bagatini et al. found that dairy protein intake improved physical function in older women (15). Finally, an insufficient consumption of protein has been associated with impairment of physical function and of quality of life in older adults with depression (12). Differently from previous studies (11–13), we found no association between exercise capacity and total protein intake, and we could not demonstrate a different physical performance between subjects ingesting more or less than 0.8 g protein per kg of body mass-daily. Several reasons may justify the contrasting results. The total protein intake of our sample (median value of 1.1 g/kg bw/day) was comparable with the intake of previous studies in Caucasians, ranging from 0.8 to 1.2 g/kg (16, 24). Thus, other factors may account for the discrepant results, as explained in the following. The difference in the results may be attributed to the fact that previous studies were generally carried out on elderly subjects (12–15), who have different skeletal muscle characteristics from those of middle-aged subjects (8). The different methods used to evaluate physical performance, directed to the measurement of strength and balance in previous studies (11–15), and to the evaluation of aerobic capacity in the present one, may also justify the contrasting results. Our data indicate that a higher vegetable protein intake is associated with a better performance at 6MWT. This result was unexpected since animal protein seems to have a higher essential amino acid content and a better protein availability, a characteristic that might ameliorate muscle protein synthesis and anabolism (7, 25). Few studies have been conducted on the separate effect of animal and vegetable protein on physical performance. Houston et al. found that the intake of animal but not vegetable protein was associated with the preservation of lean body mass in a 3-year follow-up study in older adults (16). These results were consistent with those obtained in a cross-sectional study by Lord et al. (26). In addition, the finding that an omnivorous diet increases lean body mass, while lacto-ovo-vegetarian diet results in a loss, though modest, of lean mass in males participating in a programme of resistance training, seems to support an overall better effect of animal proteins on muscle (10). After studying an older group of Chinese community-dwelling people for 4 years, Chan et al. found that a higher protein intake deriving from vegetables, but not from animal source, was associated with the preservation of muscle mass (27). Similarly, in a longitudinal cohort study in Japan, Kojima et al. showed that the age-related decline in muscle strength in women was lower in those who frequently eat soy products or green and yellow vegetables (28). The reasons for our unexpected results are not clear. The association between plant-based proteins and the 6-min walking distance may not be due to the effect of these macro-nutrients on muscle, but it could be related to other components of vegetable foods, such as antioxidants, potentially affecting muscle mass and strength (29). This hypothesis is speculative and warrants further investigation. The present study differs from the previous ones inasmuch the physical performance was evaluated by using the 6MWT, which is influenced not only by muscle strength, but also by cardiovascular and respiratory function (30). While animal proteins seem to improve muscle protein synthesis more than plant proteins (31), a vegetable protein-dietary intake is associated with beneficial cardiovascular effects both in healthy subjects (32) and in patients (33). Particularly, vegetarian dietary practices have been associated with several health benefits, among which are lower risks of dyslipidaemia, hypertension, obesity (34–37) and of chronic diseases in general (32, 38–45). Since many factors related to cardiovascular diseases may negatively affect physical performance (46), it is possible to speculate that the positive association between vegetable protein intake and 6MWD is mediated by a general health benefit rather than through a direct effect on muscle. In other words, a diet rich in vegetable products may be part of a healthy lifestyle and as a consequence of a better physical performance.

The increase in the distance walked by the intake of 10 g/day of vegetable proteins is 20 m in the adjusted model. As expected, this increase is quite small; however, there are no indications on a 6MWD variation describing a meaningful change of performance. Moreover, it is interesting to observe that we found an increase in the distance walked by 10 g/day of vegetable proteins (20 m) comparable to the difference associated with gender (adjusted difference in males with respect to females is 25.3 m). Therefore, we suppose that the increase observed is not negligible.

Our study has some limitations. Dietary assessment by FFQ is subject to the recall bias, so that the measurement error may distort the association between nutrient intakes and outcome measures. On the other hand, the FFQ provided visual aids for the estimation of portions, and this could have been useful to improve the accuracy of the reported information. Moreover, the diet was assessed at a single point in time, a fact that reflects recent rather than long-term exposure; however, there is evidence that adults maintain relatively stable long-term dietary habits (47). We also acknowledge that the study was performed on subjects without respiratory diseases, so the results could not be generalised.

The strengths and originality of this study are that we evaluated a representative sample of middle-aged subjects from the general population and we considered the vegetable and animal protein intake separately, using validated food frequency questionnaires with a careful dietary assessment.

## Conclusions

In conclusion, our population-based study did not show any association between total and animal protein intake and the distance walked in 6 min. On the contrary, a higher protein intake from a vegetable source resulted in a better physical performance. Whether this result is related to the high vegetable protein intake itself or is a consequence of the antioxidant property of plant-based foods or of some beneficial effect associated with a plant-rich dietary pattern, deserves further investigation. However, recommending higher intakes of vegetable protein might be a useful measure for ameliorating physical performance in the general population.

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## Conflict of interest and funding

The authors declare no potential conflicts of interest.

## Ethical standards disclosure

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Comitato Etico per la Sperimentazione – Azienda Ospedaliera di Verona. Written informed consent was obtained from all subjects.

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