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Environmental quality, physical characteristics and physical activities in oxidative stress induction in humans having different ages, lifestyles and working conditions. Tools and molecular epidemiology techniques for the best health promotion strategies

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ABSTRACT

Oxidative stress occurs at cellular level when the delicate equilibrium between pro-oxidants and anti-oxidants is interrupted. Although oxidative stress does not represent a pathology per se, it is involved in the pre-clinical stages of several diseases, thus its investigation is crucial from a preventive standpoint. A plethora of factors are able to affect oxidative stress, belonging to three main domains, namely the individual, the environmental and the behavioural domain. The general aim of this PhD thesis was to investigate the association between some risk factors and the oxidative stress induction in humans having different ages and lifestyles. Study lines I and II involved three sample populations of (i) 330 children (8-11 yrs.) from Asti; (ii) 205 adolescents (10-13 yrs.) from Turin and (iii) 223 subjects (9-17 yrs.) from Chivasso. Because of the COVID-19 pandemic, a systematic review on oxidative stress induction in adults was carried out to implement the study line III instead of starting a new sampling campaign. All subjects, or their legal tutors, subscribed an informed consent to participate in the studies. Then, each subject filled a standardised questionnaire gathering general information, residential address, parental education, physical activity frequency, etc. Along with the survey, the participants provided a sample of spot urine that has been stored at -80°C until analyses. The biological analyses implied the quantification of (i) the isoprostane (15-F2t-IsoP), a reliable biomarker of systemic oxidative stress in vivo; (ii) the cotinine, a metabolite of nicotine widely used to objectively assess the exposure to tobacco smoking, which is an important oxidative stress inductor; (iii) Glucuronic Acid Bisphenol A (GlcA-BPA) that has been quantified in the urinary samples as biomarker of exposure to bisphenol A ; and (iv) urinary creatinine, a physiological parameter used to normalise the aforementioned biomarkers to the excretion rate. Several independent variables have been selected and investigated. First, obesity was assessed by the Body Mass Index (BMI), obtained from the weight and height squared ratio and categorised by three BMI international reference standards, namely IOTF, CDC and WHO. Second, the exposures to surrounding green spaces (greenness) and the abovementioned bisphenol A were characterised. Greenness was defined using two metrics derived from satellite-images and calculated within individual residential buffers (built around the geocoded home-address). Additionally, using the tree census map, the residential greenness was qualified in terms of broadleaf and evergreen trees. Third, appropriate statistical analyses were performed based on distributions and associations between the variables of interest: a Generalised Linear Model was used to assess the association between BMI-based obesity and oxidative stress, a General Mixed Model for the association of oxidative stress and greenness, a Piecewise model for bisphenol A exposure and oxidative stress, and a meta-analysis to pool the summary result of the systematic review on exercise-induced oxidative stress biomarkers. The main findings are summarised as follows (i) BMI-based obesity is associated with increased oxidative stress in children and the IOTF seems to be more accurate in categorising children as obese compared to the other BMI reference standards; (ii) even a low-grade exposure to passive tobacco smoking is able to induce oxidative stress increase in healthy children; (iii) greenness exposure is associated with decreased oxidative stress in children, and physical activity could partly mediate this relationship; (iv) the exposure to bisphenol A can induce oxidative stress from a specific exposure level (6 ng/mg Crea of GlcA-BPA); (v) the quantification of a set of oxidative stress biomarkers instead of just one is advisable when investigating exercise-induce oxidative stress and (vi) moderate physical activity seems to be more effective in reducing oxidative stress levels in adults compared to strenuous exercise.

PREFACE

Primary prevention strategies are fundamental to reduce the impact of chronic diseases worldwide, acting on the environmental, societal, institutional and individual levels. Primary prevention focuses also on preventing the exposures to pollutants which may contribute to get worse the health conditions. Current scientific evidence demonstrates that, among the leading causes of death, the Non-Communicable Diseases are contributing over 70% by increasing the global burden of disease. Primary prevention has a strong interest in developing new strategies to prevent these illnesses, basing its programmes on a solid scientific basis and research in Public Health. Preventing strategies involve several modifiable, or partly modifiable, factors such as lifestyle and environmental features, whose modification may remarkably impact health and well-being.

Acting before the outbreak of diseases captures the intimate essence of the primary prevention and, in this context, more efforts should be made to understand the initiation pathways that lead to future pathologies. Oxidative stress and inflammation are two reciprocally linked processes which may contribute to the onset or exacerbation of many human diseases, if not properly monitored and counteracted.

The concept of oxidative stress has been introduced in redox biology and medicine in 1985, defined as "*an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage*" (Sies 2015). Although oxidative stress does not represent a pathology itself, it may contribute in the detection of risky conditions and to know pathogenesis and progression of several chronic diseases.

This PhD thesis gives insight into the oxidative stress quantification in response to various *stimuli* and conditions, supporting applied research in primary prevention and contributing to increasing the body of evidence with a molecular epidemiology approach.

The current PhD thesis was developed between 2017 and 2020 in the contest of a doctoral research project carried out under the mentorship of Prof. Roberto Bono at the Department of Public Health and Pediatrics of the University of Turin. It consists in a compilation of three study lines and five papers (two papers already published, one under-review and two to be submitted) co-authored by the PhD candidate.

The five papers described in the current thesis were aimed at investigating the oxidative stress induction due to some key factors belonging to three main domains, namely the **individual**, **environmental** and **behavioural** domain. The specific objectives were (i) testing the association between obesity (assessed by the Body Mass Index "BMI") and oxidative stress in children; (ii) investigating whether, among three selected international BMI reference standards, one of them was more accurate in categorising children as overweight and obese; (iii) testing the association between the exposure to green spaces and oxidative stress in children and adolescents; (iv) spatialising the association (if present) between oxidative stress and green spaces in order to identify the city areas at "higher risk"; (v) explore the non-invasive biomarkers of oxidative stress induced by physical activity in adults.

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15-F2t-IsoP: Isoprostane
15-F2t-IsoP-Ev: Isoprostane Envelope
8-OH-dG: 8-hydroxy-2' -deoxyguanosine
8-oxo-dG: 8-oxo-7,8-dihydro-20-deoxyguanosine
a.s.l.: above sea level
BF: Body Fat (%)
BIA: Bioelectric Impedance Analysis
BMI: Body Mass Index
BPA: Bisphenol A
CDC: Centers for Disease Control and Prevention
COPD: Chronic Obstructive Pulmonary Disease
Crea: Creatinine
DNA: DeoxyriboNucleic Acid
EFSA: European Food Safety Authority
ELISA: Enzyme-Linked ImmunoSorbent Assay
GC: Gas Chromatography
GlcA-BPA: Glucuronic Acid of Bisphenol A
GLM: Generalised Linear Model
HPLC: High Performance Liquid Chromatography
IOTF: International Obese Task Force
IQR: Inter Quartile Range
ISGlobal: Instituto de Salud Global
LC: Liquid Chromatography
LMM: Linear Mixed Model
LOD: Limit Of Detection
LOQ: Limit Of Quantification
LRM: Linear Regression Model
MS: Mass-Spectrometry
mSAVI: masked SAVI
NBT: Number of Broadleaf Trees
NBT-Ev: Number of Broadleaf Trees Envelope
NDVI: Normalised Difference Vegetation Index
NET: Number of Evergreen Trees
NET-Ev: Number of Evergreen Trees Envelope
NIH: National Institute of Health
NIR: Near-Infrared
NW: Normal-Weight
OB: Obese
OW: Over-Weight
PGC: Percentage of Green Cover
PGC-Ev: Percentage of Green Cover Envelope
PUFAs: Polyunsaturated Fatty Acids
REML: Restricted Maximum Likelihood
RNA: RiboNucleic Acid
ROS: Reactive Oxygen Species
SAVI: Soil Adjusted Vegetation Index
SD: Standard Deviation
SIM: Selected Ion Monitoring
SMD: Standardised Mean Difference
uSAVI: unitary Soil Adjusted Vegetation Index
uSAVI-Ev: unitary Soil Adjusted Vegetation Index Envelope
UW: Underweight
WHO: World Health Organization

1. GENERAL INTRODUCTION

1.1. Oxidative stress

The term "oxidative stress" was first coined in 1985 by Dr Helmut Sies who originally introduced it as "*a disturbance in the prooxidant-antioxidant balance in favour of the former*" (Sies 2015). In 2007, an up-to-date definition of oxidative stress was formulated as follows: "*an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage*" (Sies 2018). From the last definition, oxidative stress has been linked to an intensity-related dichotomy where low exposure to oxidative stress serves as positive redox signalling in cells (i.e. "*eustress*") and high exposure may lead to an unspecific biomolecular damage, causing "oxidative distress", thus health impairments (Sies 2018). This dichotomy calls to mind a universal biological concept: the homeostasis. In the 19th century, Dr Claude Bernard, a French physiologist, firstly introduced the relevance of maintaining the "*milieu intérieur*", which we know today as the internal environment (Ursini et al. 2016). The loss of homeostasis may lead to injuries and diseases, therefore preserving or restoring it is a well-conserved mechanism in the evolution. In biological terms, maintaining or restoring the redox homeostasis is a similar phenomenon, that involves sophisticated regulatory or adaptative processes, based on a signal delivering through redox chemistry (i.e. redox signalling) (Dröge 2002). In fact, the stability of the internal homeostasis is associated with many different rhythms (e.g. circadian) and referring to an "allostatic buffering capacity" (Li and He 2009) might integrate the concept of the homeostasis. The allostatic buffering capacity refers to dynamic stability rather than a fixed equilibrium by which each living organisms has a certain range of adaptation (i.e. buffer), within the physiologic variations. Redox signalling is widely used by living organisms to induce protective responses, control cell growth, differentiation, and death (Franco et al. 2019). Redox homeostasis is continuously maintained or rearranged by: (1) the redox signalling; (2) the hormesis, which is a biphasic adaptative response able to establish a new homeostatic setting (Calabrese and Baldwin 2002; Ursini et al. 2016). However, when a stable and prolonged perturbation of homeostasis occurs and nor the redox signalling, neither the adaptation through the hormesis are efficient in its restoration, oxidative stress is established. In other terms: oxidative stress emerges as the consequence of a marked redox homeostasis perturbation. However, it does not represent a pathology itself since living organisms can both restore or rearrange the homeostasis, but it represents a condition of risk which may lead to pathological conditions, if cells defences are overwhelmed.

1.1.1. Background concepts

The major role of oxygen for all aerobic organisms is to act as a sink or dumping ground for electrons that are the chemical fuel for cell metabolism, making oxygen essential for life (Davies 1995). The paradox of aerobic life is that oxygen has also a potentially damaging side-effect, due to the production of highly reactive by-products during oxidative phosphorylation reactions. Therefore, cells and organs are continuously exposed to oxidants, which are formed under physiological conditions by the aerobic metabolism. Reactive Oxygen Species (ROS) are produced by living cells as result of cellular aerobic metabolism from molecular oxygen (Birben et al. 2012). The oxygen molecule undergoes four-electron reduction during its *in vivo* metabolization (Yoshikawa and Naito 2002), which generates ROS. These chemical species are defined as highly reactive by-products of oxidation processes and may be characterised by the presence of one or more unpaired electrons in their outer shells (i.e. radical species or free radicals), or may be nonradical reactive species.

Reactive Oxygen Species		Formula
Radical	Superoxide anion	$O_2^{\bullet-}$
Non radical	Hydrogen peroxide	H_2O_2
Non radical	Singlet oxygen	$^1O_2$
Radical	Hydroxyl radical	$^{\bullet}OH$

Table 1 *Major Reactive Oxygen Species in biological systems*

The superoxide anion ($O_2^{\bullet-}$) formation is mediated by Nicotine Adenine Dinucleotide oxidase and xanthine oxidase enzymes or is formed nonenzymatically by the semi-ubiquinone compound within the mitochondrial electron chain (Dröge 2002). The major production sites of the superoxide anion, are mitochondria, cellular organelles responsible for energy production in eukaryotic cells by the electron transport chain that reduces oxygen to water. Under physiological conditions, this process is not totally efficient resulting in about 2% of oxygen converted into superoxide anion rather than water (Burton and Jauniaux 2011). The superoxide anion can be converted, by superoxide dismutase enzymes, into hydrogen peroxide (H_2O_2) and, in biological tissues, into singlet oxygen ($^1O_2$) by nonenzymatic reactions (Deby and Goutier 1990; Fridovich 1978). Additionally, in presence of transition metals in reduced forms (e.g. ferrous iron Fe^{2+}), it generates hydroxyl radicals ($^{\bullet}OH$) (Chance et al. 1979). The hydroxyl radical is the most reactive among ROS (Betteridge 2000) and reacts close to its formation site due to the extremely short-life (Burton and Jauniaux 2011).

Given the reactivity of ROS, living organisms have evolved antioxidants defence systems to counterbalance the effect of oxidants. These molecules are able to re-establish the redox homeostasis after a temporary increase of ROS. Based on their nature or action, antioxidants have been classified as follows: (1) *enzymatic antioxidants*, including superoxide dismutase, catalase, glutathione peroxidase/reductase; (2) *non-enzymatic and nutrient antioxidants* such as alpha-tocopherol (vitamin E), ascorbic acid (vitamin C) and beta-carotene; and (3) *metabolic antioxidants*, namely glutathione, bilirubin, uric acid, transferrin, caeruloplasmin, albumin and haptoglobin (Hasan 2017). Figure 1 summarises both ROS production and clearance within biological systems.

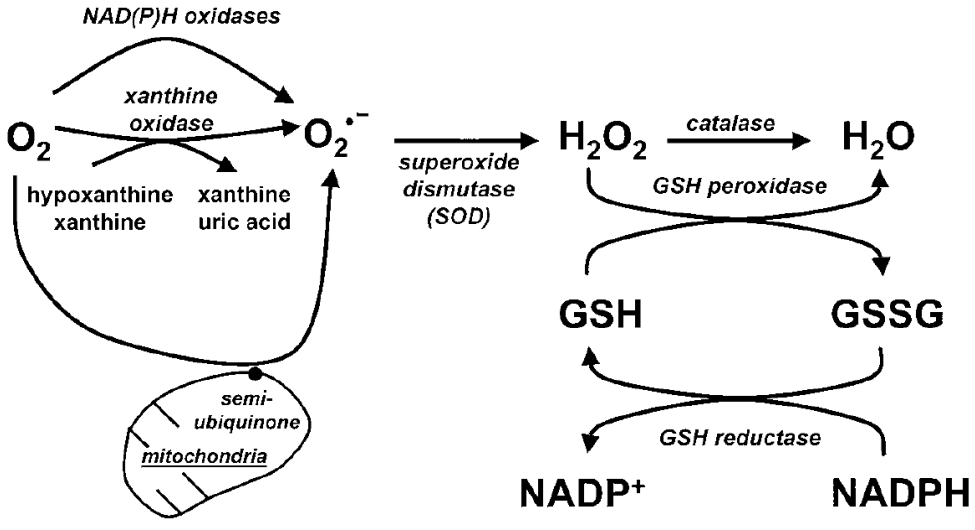


Figure 1 *Pathways of Reactive Oxygen Species production and clearance (Dröge 2002)*

Despite their toxic effect, ROS have several important physiological functions in living organisms and only their uncontrolled overproduction promotes redox disturbances leading to oxidative stress. From an evolutive perspective, aerobic organisms have evolved trying to counteract the ROS toxicity while taking advantage from their biological effects. Indeed, at homeostatic levels or within the

aforementioned *eustress*, ROS participate in numerous biological mechanisms. First, play a central role in the intracellular signalling cascade in various types of cells, activating specific transduction pathways (e.g. amino acid cysteine of proteins, protein kinases or phosphatases, etc.) that rule homeostasis, growth, autophagy and apoptosis (Lushchak 2014; Milkovic et al. 2019). Second, ROS are involved in the immunological modulation by amplifying the immune responses of lymphocytes, by inducing the adherence of leukocytes to endothelial cells (Dröge 2002) and by contributing in the "immunological burst" of macrophages and phagocytes in first line defence (Das Sarma et al. 2010). Third, ROS participate in guiding stem cells 'molecular mechanisms of differentiation (Roy et al. 2017; Sena and Chandel 2012). Finally, they are involved in skeletal muscle contractions, driving the modulation and the adaptations of muscle to exercise (Roy et al. 2017).

1.1.2. Oxidative Stress effect on biomolecules and cells

The physiological roles of ROS into biological systems do not make oxidants harmless. As previously mentioned, at high concentrations free radicals, radical-derived and nonradical reactive species can lead to oxidative stress. This latter, is able to damage all major cellular constituents, implying alterations in the chemical-physical properties of the biomolecules (e.g. inhibiting their functions or damaging the functionality of cells and tissues). ROS can alter the structure and function of proteins inducing their dimerization (Butnariu 2012), and the direct oxidation of amino acids (Burton and Jauniaux 2011). They attack nucleic acids (RNA and DNA) causing bases degradations, bonds breaks, mutations and cross-linking phenomena (Birben et al. 2012; Cadet et al. 2017). Further, ROS damage lipids by free-radical mediated lipid peroxidation reactions. In particular, the unsaturated double bonds of Polyunsaturated Fatty Acids (PUFAs), make these essential lipids susceptible to ROS attack (Marrocco et al. 2017). Free radical-mediated lipid peroxidation occurs by removing a hydrogen atom from the fatty acid that composes the cellular membrane, mainly linoleic and arachidonic acids (Liguori et al. 2018; Yoshikawa and Naito 2002). This chain reaction transforms PUFAs into lipid hydroperoxides, which are highly unstable and easily decompose to secondary products (e.g. aldehydes, malondialdehydes and isoprostanes) (Birben et al. 2012) that are commonly measured as proxy of oxidative stress. The lipid peroxidation leads to the cellular membrane structure rearrangement, loss of fluidity and even to the activation of the apoptotic cascade (Burton and Jauniaux 2011).

1.1.3. Oxidative Stress effect on human health

According to the "free-radical theory of aging", first postulated by Dr Harman in 1956 (Harman 1956) and afterward modified into "oxidative stress theory" (Ghezzi et al. 2017), ROS are intimately involved in the ageing processes. The term "ageing" refers to that intrinsic and degenerative process of living organisms accompanied by a progressive loss of function and fitness rather than the mere march of time. Theoretically, the accumulation of oxidative damage in macromolecules over time can affect lifespan (Belenguer-Varea et al. 2020) and health, being associated to the immune system dysregulation and to the increase of inflammation mediators (i.e. inflammatory processes) (Tan et al. 2018). The mechanisms underlying oxidative stress and ageing are still not clear, however the decline in the efficiency of antioxidant defence systems in elderly people could partly explain them (Islam 2017) .

Oxidative stress has been implicated in the pathophysiology of numerous pathologies, whose incidence is higher in old people but not restricted to the elderly-hood, including (1) **neurodegenerative diseases**, such as Parkinson and Alzheimer diseases, amyotrophic lateral sclerosis, multiple sclerosis and also dementia; (2) **cardiovascular diseases**, including atherosclerosis, ischemic heart diseases, hypertension, cardiomyopathies, and even cardiac hypertrophy; (3) **respiratory diseases**, predominantly Chronic Obstructive Pulmonary Disease (COPD) and asthma; (4) **renal diseases**, including acute and chronic kidney diseases, glomerulo and tubule-interstitial nephritis, renal failure, proteinuria and uraemia; (5) **cancer**, where chronic

inflammation induce oxidative stress in a vicious cycle that feeds itself causing further chemical and structural alterations and (6) other diseases including **diabetes**, **rheumatoid arthritis**, **sarcopenia** and **frailty** (Liguori et al. 2018; Pizzino et al. 2017; Roy et al. 2017).
It has been speculated that oxidative stress is associated to the aetiology of these pathological conditions as it was observed in their early stages (Czerska et al. 2015). By contrast, the redox disequilibrium might be considered only a downstream consequence of the pathologies (Roy et al. 2017). Irrespective of whether oxidative stress is a cause or a consequence of the pathological condition, its measurement may support Public Health either in prevention strategies either in understanding the progression of certain diseases.
Of note, as recently as 2020, a systematic analysis published on Lancet (Vos et al. 2020), reported the global burden of 369 diseases and injuries in 204 countries, highlighting that the ten most important drivers of increasing burden between 1990 and 2019 were (1) ischemic heart diseases; (2)diabetes; (3) stroke; (4) chronic kidney diseases; (5) lung cancer; (6) age-related hearing loss; (7) HIV/AIDS infections; (8) musculoskeletal disorders; (9) low back pain and (10) depressive disorders. Oxidative stress has been reported to be involved in six out of the ten abovementioned diseases, namely ischemic heart diseases (Roy et al. 2017), diabetes (Baynes 1991), chronic kidney diseases (Balasubramanian 2013), lung cancer (Filaire et al. 2013), HIV (Eck et al. 1989), and depressive disorders (Bhatt et al. 2020).

1.2. Biomonitoring oxidative stress

The field of molecular epidemiology exploits biomonitoring to investigate the aetiology, genetic susceptibility and mechanisms that are implicated in diseases. A key role lies in the usage of biomarkers to foresee the disease development or progression, and to implement targeted prevention programs. Hence, a major objective of molecular epidemiology is to provide reliable and specific information regarding the aetiology of pathological processes for disease prevention (Bonassi and Au 2002) using human biomonitoring. Human biomonitoring is defined as "*the repeated, controlled measurement of chemical or biochemical markers (i.e. **biomarkers**) in fluids, tissues or other accessible samples from subjects who are/were/will be exposed to chemical, physical or biological risk factors in both the occupational and general environment*" (Ladeira and Viegas 2016). It may reflect specific exposures aiming at predicting risky conditions to tailor better prevention strategies in Public Health. Moreover, biomonitoring data support the protection of susceptible populations (Angerer et al. 2007; Den Hond et al. 2015), including children, pregnant women and elderly. In general terms, a biomarker identifies "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention*" (Atkinson et al. 2001). The quantification of biomarkers may directly reflect the burden of biological effects resulting from all exposure routes as well as the inter-individual variability. Ideally, a suitable biomarker should be accessible, stable and non-destructive, while its measurement should be safe, cost-effective and easy to perform. For this reason, non-invasive biomarkers, such as those measured in exhaled breath, saliva and urine, are widely used especially in large-scale studies. Additionally, urine is a better matrix compared to other specimens (e.g. plasma or serum) to quantify oxidative stress because it has much lower oxidizable organic and inorganic content (Il'yasova et al. 2012).
Biomarkers are commonly grouped into three classes, although in certain situations the definitions partly overlap: (1) **biomarkers of exposure**, including exogenous substances, their metabolites or even derived adducts that can reflect the internal dose, the biologically effective dose or the target dose of specific xenobiotics; (2) **biomarkers of effect**, defined as measurable structural, functional or biochemical alteration, which reflects early reversible changes in the organism (e.g. altered expression of metabolic enzymes or early pathological changes such as mutations and lesions); and (3) **biomarkers of susceptibility** that refers to the individual inherent or acquired ability to respond

to specific perturbations due to external exposures (e.g. enzymes involved in the xenobiotic biotransformation, oncogenes, etc.) (Manno et al. 2010; Silins and Högberg 2011).
In the context of oxidative stress biomonitoring, it is common practice to measure some oxidation by-products or metabolites (Monaghan et al. 2009) rather than ROS themselves. These chemical species are derived from ROS-mediated oxidation reactions of macromolecules and cells. The assumption underlying the indirect measurement of oxidative stress is that oxidation derivates are proportional to the ROS extent (Il'yasova et al. 2012) and, at the same time, they are more stable, thus detectable in the body fluids. In the current thesis only a small number of oxidative stress biomarkers will be presented depending upon the original results of this doctoral project.

1.2.1. Biomarker of lipid peroxidation: isoprostane (15-F2t-IsoP)

Isoprostanes are stable and specific by-products of oxidation-induced lipid peroxidation *in vivo*. They are prostaglandin-like compounds mainly generated by the non-enzymatic oxidation of the arachidonic acid, independent of cyclooxygenase. Unlike prostaglandins, isoprostanes are formed non-enzymatically (Milne et al. 2008), which partly circumvents the issue of enzyme-derived variability (Il'yasova et al. 2012). Moreover, isoprostanes are formed *in situ* on the lipid membranes (Czerska et al. 2015) and may also derived from the peroxidation of docosahexaenoic and eicosapentaenoic acids (Jadoon and Malik 2018). Isoprostanes are considered the most reliable biomarkers of oxidative stress *in vivo* (Frijhoff et al. 2015). They are detectable in a large set of biological fluids and are not affected by the lipid content of diet (Roberts and Morrow 2000). Depending on the position where the oxygen molecule is added to the arachidonic acid, four regioisomeres are formed, comprising 16 stereoisomers each (Il'yasova et al. 2012). **15-F2t IsoP** is one of the most extensively studied isoprostane isomers (Basu 2008; Graille et al. 2020), used as proxy for the change of general isoprostane levels (van 't Erve et al. 2017). 15-F2t IsoP is also the most commonly used oxidative stress biomarker in human studies (Ito et al. 2019) . This biomarker of effect (Milne et al. 2015) exerts powerful vasoconstrictor properties, is able to modulate the activity of platelets, inhibit angiogenesis and promote atherogenesis (Milne et al. 2015).

1.2.2. Biomarker of DNA oxidation: 8-hydroxy-2' -deoxyguanosine (8-OH-dG) and 8-oxo-7-hydro-2'-deoxyguanosine (8-oxo-dG)

The interaction between ROS and the nucleobases of the DNA strand, results in the generation of several DNA adducts and lesions. Guanine is the most easily oxidized nucleobase of DNA, due to its low redox potential (Steenken and Jovanovic 1997). When the hydroxyl radical is added in the C8 position of guanine, the formation of C8-hydroxyguanine adduct occurs. The C8-hydroxyguanine adduct may undergo further reactions such as the reduction to 7-hydro-8-hydroxy-2'-deoxyguanosine, or the oxidation to both 8-hydroxy-2'-deoxyguanosine (8-OH-dG) or its tautomer 8-oxo-7-hydro-2'-deoxyguanosine (8-oxo-dG) (Valavanidis et al. 2009a). The oxidative modifications of DNA may lead to mispairing, since they can pair with guanine nucleobases causing GT →TA transversion (Frijhoff et al. 2015), thus mutations. However, the oxidised nucleosides are repaired by excision and then excreted in the urine, where are measured as a biomarker of oxidative stress. Indeed, the urinary concentration of 8-oxo-dG and 8-OH-dG reflects the rate at which DNA is both oxidized and repaired (Il'yasova et al. 2012), introducing a substantial inter-individual variability (Wilson et al. 2011). These nucleosides have been validated as oxidative stress biomarkers in animal models (Kadiiska et al. 2000) and, in particular the 8-oxo-dG lesions which are daily formed in mammalian cells, represent 5% of all oxidative lesions (Marrocco et al. 2017). Cell death and diet are not able to considerably affect the urinary levels of 8-oxo-dG (Cooke et al. 2008).

1.3. Sources of oxidative stress

Oxidative stress biomarkers are presumed to increase in the "pre-clinical stages of diseases" because of the influence of a plethora of factors (Graille et al. 2020), which are able to increase ROS

generation directly or indirectly. Oxidative stress may be induced by occupational or environmental exposures, and by unhealthy lifestyles or behaviours. For example, exogenous sources include (1) environmental exposures, such as UV radiation (de Jager et al. 2017), toxic agents (Zheng et al. 2020), air pollution (Gangwar et al. 2020), passive tobacco smoking (Bono et al. 2014), and air-formaldehyde (Bellisario et al. 2016; Tesfaye et al.); (2) occupational exposure, such as pesticides and metals (Soleimani et al. 2015), benzene (Moro et al. 2019), wood dust (Bono et al. 2019) and nanomaterials (GHAFARI et al. 2020) and even (3) viral infections (Ivanov et al. 2016; Zhang et al. 2019). Of note, it has been speculated that the uncontrolled oxidative stress is one of the main causes of morbidity and mortality due to the severe acute respiratory syndrome observed in patients infected by SARS-CoV-2 (de las Heras et al. 2020). Oxidative stress has been proposed as a key player in the progression and the exacerbation of the SARS-CoV-2 infection (Cecchini and Cecchini 2020; Delgado-Roche and Mesta 2020; Zarbafian et al. 2020), to the extent that the antioxidant therapy (e.g. Vitamin D and melatonin) has been proposed as promising co-adjuvant in its treatment (Beltrán-García et al. 2020).

Concerning other potential oxidative stress inductors, those modifiable refers to lifestyles and behaviours, such as active tobacco smoking, alcohol consumption, unhealthy diet, and physical activity, which can represent a pro-oxidant if carried out vigorously, while acts as an antioxidant when performed with moderate intensity. Age and sex can be considered as intrinsic, non-modifiable individual characteristics which, however, worth to be mentioned as oxidative stress influencing factors. Moreover, body composition and in particular overweight and obesity, which are partly due to lifestyles, but also depend on individual characteristics, have been linked to increased oxidative stress. Lastly, as mentioned previously, certain pathological conditions are characterised by a chronic increase of oxidative stress, which is involved in their worsening.

Several of the aforementioned categorisations should not be intended so rigid, since several oxidative stress inductors theoretically belong to a specific category, but the exposure occurs in relation to definite behaviours and lifestyles. For example, bisphenols are environmental contaminants whose exposure largely depends on consumer's choices, thus individual behaviours; in particular bisphenol S, often used as an intermediate for the production of epoxy resins and polycarbonate plastics, has been detected in food packaging, baby bottles, toys, dental materials, personal care products and papers (Wu et al. 2018).

Given the foregoing points, a large number of factors may be implicated in the induction of oxidative stress which, in turn, may lead to future diseases, if not properly counterbalanced. This is the main point from the prevention standpoint, whose pivotal objectives include the protection and promotion of health and well-being, before the pathological condition is established. Counterbalancing oxidative stress does not only include the involvement of physiological antioxidants, but also preventive strategies targeted to the reduction of risky circumstances acknowledged to cause or trigger oxidative stress.

The following paragraphs will give insight into the specific factors that have been studied as potential oxidative stress inductors within this doctoral project. Several key concepts will be presented afterwards, to support the readers in the comprehension of the original results reported by the thesis dissertation.

1.3.1. Environmental settings and contaminants: urban green spaces (greenness) and bisphenol A

Among a large set of **environmental factors and contaminants**, acknowledged or supposed inductors of oxidative stress, this dissertation mainly focused on greenness and bisphenol A.

Greenness

In the last decade, an accumulating body of evidence has shown that access and exposure to "greenness" are related to positive health effects. The term *greenness* commonly refers to green spaces in epidemiological studies, and has been linked to a reduced risk of overweight and obesity, improved mental health, higher birth weight, increased physical activity, and lower mortality rates (Fong et al. 2018; James et al. 2015). For other health outcomes, such as asthma or allergic diseases, the association with greenness is still inconsistent (Fuertes et al. 2016; Hartley et al. 2020; Lambert et al. 2017). In general, the exposure to greenness may benefit health via three main pathways or mechanisms. First, by the mitigation of other environmental risk factors, such as air and noise pollution (Dadvand et al. 2012; De Ridder et al. 2004; Wolch et al. 2014; Yang et al. 2005). Second, by modifying behaviours, since the proximity to parks and gardens, or greater amount of greenness, have been related to greater outdoor physical activity both in children and adults (Fong et al. 2018; Gray et al. 2015; Grigsby-Toussaint et al. 2011; Markevych et al. 2016; McMorris et al. 2015), which in turn would help in body weight control. Third, greenness promotes social aggregation and reduces general stress, which in turn affect the risk of other diseases (Hartig et al. 2014). To date, less is known on the direct and indirect effects of greenness on biological pathways, including inflammation and oxidative stress.

Bisphenol A

According to the European Food Safety Authority (EFSA), the general population can be exposed to bisphenol A in external, internal, and aggregated ways via food, dermal contact (e.g. cosmetics and thermal paper), drinking water, and simply breathing indoors and outdoors (Mielke and Gundert-Remy 2009). Moreover, infants can suffer an even greater exposure via breast feeding (LaKind and Naiman 2011). Bisphenol A is a synthetic organic compound extensively used in the manufacturing of polycarbonates and epoxy resins (Abraham and Chakraborty 2019). After the exposure to UV light or when bisphenol A-based plastics are placed into acidic or basic solution, they can release monomeric bisphenol A. Consequently, plastic food and drink containers can become a widespread Public Health risk (Talsness et al. 2009), largely contributing to the daily intake of bisphenols. Given the widespread exposure to bisphenol A, in 2015 the EFSA reduced the temporary Tolerable Daily Intake of bisphenol A from 50 to 4 µg/kg bw/day. This endocrine disruptor is able to induce or contribute to a large number of diseases, including reproductive, perinatal, and pediatric outcomes, and also hepatic tumours, lung inflammation, Parkinson disease, abnormal behaviour, obesity, diabetes, and reproductive abnormalities (Wang et al. 2018). Furthermore, bisphenol A can induce an increase in oxidative stress (Gassman 2017; Qiu et al. 2016; Wang et al. 2019). Glucuronic acid of bisphenol A (GlcA–BPA) is a urinary metabolite of bisphenol A, currently considered its major residue, both in vitro and in vivo (Dekant and Völkel 2008), which makes suitable the its measurement in molecular epidemiology studies. However, studies exploring the exposure to bisphenol A linked to the induction of inflammation, lipid peroxidation and oxidative stress, are still scarce (Asimakopoulos et al. 2016; Y.-C. et al. 2009).

1.3.2. Individual characteristics: age and body composition

In the context of the current thesis, two **individual characteristics**, namely age and body composition, have been investigated as oxidative stress inductors. Although previous literature has already documented their effect on oxidative status, this doctoral project investigated them from a different perspective, which will be elucidated in the results session.

Age

The growth process, although uncontrollable, must be considered a fully-fledged factor of oxidative stress induction. In fact, oxidative stress changes along with the different phases of life, not only increasing in elderly people (Andriollo-Sanchez et al. 2005), as a consequence of the antioxidant

defence systems depletion (Islam 2017), but also affecting infants, children and adolescents. Especially infants and children represent another fragile segment of the population, susceptible from numerous points of view. Firstly, they drink 2.5 times more water than adults, by the percentage of body weight (Plunkett et al. 1992), and children aged between one and five years eat 3 to 4 times more than adults, per unit of body weight. Secondly, their brain and nervous systems develop until adolescence, thus they are extremely sensitive to detrimental exposures due to this critical development stage (Rodier 1995). Thirdly, infants and children are not able to metabolise, detoxify and excrete toxins as efficiently as adults (Echobichon and Stevens 1973). Finally, children breathe more rapidly, (e.g. new-borns take 60 breaths per minute versus 12 in adults), therefore they breathe a larger air volume per minute compared to adults (Bearer 1995). Consequently, infants and children are likely to be exposed to greater quantities of xenobiotics via breathing and eating, thus they can be considered more prone to xenobiotics-induced oxidative stress.

Body composition

Overweight and obesity are an emerging health issue worldwide, especially in the paediatric population (Bahreynian et al. 2018; Vecchio et al. 2018). In the time-lapse between 1975 and 2016, their prevalence, among children and adolescents, has risen from 4% to over 18% (World Health Organization 2016). Childhood obesity is associated with higher future risks of obesity, premature death, and disability in adulthood. Indeed, obesity in youth predisposes children to chronic adulthood health problems, such as metabolic syndrome, diabetes mellitus, cardiovascular diseases, and cancer (World Health Organization 2016). The excess of adipose tissue has been identified as a source of pro-inflammatory cytokines (Fonseca-Alaniz et al. 2007) ,which stimulate ROS accumulation, by increasing the lipid peroxidation rate (Khan et al. 2006). Hence, the adipose tissue in obese subjects determines an increase of local and systemic production of pro-inflammatory adipocytokines nourishing the oxidative stress induction (Khan et al. 2006) and a low-grade inflammatory response (Marseglia et al. 2014; Rzheshevsky 2013). Consequently, the early identification of the excess of body fat is essential in the paediatric population to prevent future diseases in adult life (Alves Junior et al. 2017). Several techniques have been employed for the definition of body composition, including Dual-energy X-ray Absorptiometry, computed tomography, Magnetic Resonance Imaging, and Bioelectrical impedance Analysis (BIA). However, they are operationally-costly and/or require expensive training (Alves Junior et al. 2017). Thus, alternative methods with low operational costs, that can be easily used in large-scale studies and with children, have been used in epidemiology. For example, Body Mass Index (BMI) is a descriptive index of body habitus, used to this purpose. A significant advantage of BMI is the availability of national references and its established relationships with levels of body fatness, morbidity, and mortality in adults (Duren et al. 2008). However, different age-dependent cut-offs have been adopted by each specific reference standard and consequently children classifications as “underweight” (UW), “normal-weight” (NW), “overweight” (OW) and “obese” (OB) varies based on the reference standard used.

1.3.3. Lifestyles: physical activity and tobacco smoking

Lifestyle modification is the foundation of the primary prevention strategies. Altering individual habits over time, such as diet, physical activity, alcohol and tobacco consumptions, may prevent or help the managing of several diseases. The current thesis took into account physical activity and tobacco smoking as valid example of **lifestyles and modifiable factors** able to induce oxidative stress, thus to affect human health.

Physical activity

In 1978, physical exercise was associated with the increase of oxidative stress for the first time (Fisher-Wellman and Bloomer 2009). During physical activity, the organism is exposed to various types of stress (e.g. metabolic, hypoxic, mechanical, oxidative etc.) that act as homeostatic perturbations (Peake et al. 2015). Indeed, these stresses can stimulate the biochemical signalling pathways, signifying an acute increase of ROS, which can lead to oxidative stress, if the antioxidant systems are overwhelmed. On the other hand, a sedentary lifestyle has been associated with several diseases, such as obesity, type 2 diabetes mellitus, hypertension and many others (de Sousa et al. 2017). Thus, based on the intensity (Goto et al. 2003) and duration (Fisher-Wellman and Bloomer 2009), physical activity may act either as an antioxidant stimulus or as an oxidative stress trigger. Acute bouts of physical activity have been linked to increased oxidative stress (Fisher-Wellman and Bloomer 2009). However, moderate and regular physical activity is able to enhance muscle oxidative capacity (Li and He 2009), muscle force production (Powers et al. 2016) and general antioxidant responses acting as an adaptative stimulus against excessive oxidative stress (Powers et al. 2016; Radak et al. 2005; Webb et al. 2017). Regardless of the current evidence related to this topic, the quantification of exercise-induced oxidative stress biomarkers is still challenging, especially in large-scale studies where the cost-effectiveness and non-invasive nature of measures are crucial.

Tobacco smoking

Epidemiological studies have recognised smoking as one of the most important environmental causes of human mortality and morbidity, accounting for approximately 30% of all cancer deaths in developed countries (Vineis et al. 2004). Moreover, tobacco smoking causes a greater number of deaths from cardiovascular, chronic obstructive pulmonary and degenerative diseases (Valavanidis et al. 2009b).

Tobacco smoking is a complex mixture containing more than 5,000 chemicals (Talhout et al. 2011), most of them with toxic and carcinogenic properties. It has been estimated that each cigarette puff contains 10^{14} free radicals (Pryor et al. 1983). The oxidants content in the gas phase primarily affects the upper respiratory tract, due to its short half-life, while the tar phase oxidants are relatively more stable, thus may be even more detrimental, especially in the lungs (Seagrave 2000). As previously mentioned, an overproduction of ROS can cause injuries to lipids, proteins, carbohydrates, and DNA, via oxidative damaging. Tobacco smoking is an established risk factor for several diseases, including asthma, atherosclerosis, rheumatoid arthritis, psoriasis, and cardiovascular diseases and many others (Foronjy and D'Armiento 2006; Kaur et al. 2019; Milnerowicz et al. 2015; Strzelak et al. 2018). Moreover, smoking increases the risk of several types of cancer, namely lung, mouth, larynx, bladder, kidney, pancreas, and uterine cervix cancers. One of the underlying mechanisms linking cigarette smoking and health impairments is oxidative stress. Tobacco smoking is a recognised inductor of oxidative stress, since it can induce the free-radicals production and the activation of the inflammatory immune response (Bellisario et al. 2019; Foronjy and D'Armiento 2006). Existing evidence suggests that both passive and active tobacco smoking affect health (Cao et al. 2015). Passive tobacco smoking exposure leads to the onset of respiratory symptoms, most commonly cough and eye irritation and has been linked to COPD, bronchial asthma, cardiovascular diseases, and higher incidence of neoplasms, in adults (Accordini et al. 2018); while lower respiratory tract infections and a reduced lung function have been observed in children (Hofhuis et al. 2003).

Based on the abovementioned evidence, both active and passive tobacco smoking exposures have been considered as important confounding factors in the oxidative stress induction investigated in the current thesis.

2. HYPOTHESIS

Several factors, whose domains are disparate relying on individual characteristics, surrounding environment, and behaviours may influence oxidative stress both directly/indirectly. Further, all these different domains should be considered either separately either jointly, as feasible. In particular:

Individual domain. As previously mentioned, different growing phases are supposed to affect oxidative *status*. Hence, we hypothesised that the analysis on differently-aged populations could (i) exemplify the investigation on oxidative stress induction due to other factors than age itself and (ii) support the comprehension of the variable "age" when it is considered as confounder or covariate in multivariable analyses. We also hypothesised that if BMI is used as proxy of body composition in children, particular attention should be paid in assessing obesity-related oxidative stress. Children classified as obese, according to their BMI, may have higher oxidative stress compared to normal-weight children. However, the availability of several international BMI reference standards, which allocate children in the specific group relying on diverse cut-off values, may imply different sensitivity and specificity for each BMI reference standard. Therefore, there could be a BMI reference standard with greater accuracy in categorising children with the final purpose of clarifying obesity-related oxidative stress;

Environmental domain. Environmental features may indirectly affect health by modulating oxidative stress. However, this domain shows great complexity, comprising a large number of factors, which cannot be always measured or controlled, especially in observational studies. Therefore, we deliberately chose some key features that could be intended as an exemplification of the environmental conditions and, at the same time, could provide an example of different underlying mechanisms linked to oxidative stress. In general, we hypothesised that the degree of urbanisation could play an important role in oxidative stress induction. In particular, urban green spaces may be beneficial in lowering oxidative stress biomarkers by modifying behaviours (e.g. physical activity) or by mitigating detrimental exposures (e.g. air pollution). Indeed, environmental exposure to airborne chemicals, such as bisphenol A, may directly affect general and respiratory health by increasing oxidative stress and inflammation.

Lifestyles domain. Behaviours and lifestyles can affect health by modulating oxidative stress acting both as predictors and/or confounders (e.g. tobacco smoking exposure, physical activity). We hypothesised that physical activity frequency could modulate oxidative stress in either positive and negative directions and its role, especially in relation to outdoor facilities or circumstances, should be considered. On the other hand, tobacco smoking exposure, as another example of modifiable factor, has been hypothesised to affect oxidative stress even when passive and at low doses.

3. OBJECTIVES

3.1. General objectives

This PhD thesis aims to the implementation of the body of evidence on potential or acknowledged factors affecting oxidative stress. Therefore, specific risky conditions and different sample populations have been considered and investigated using a molecular epidemiology approach.

The epidemiological approach serves properly this scope and provides useful data for planning new prevention strategies in Public Health. External and internal *stimuli* impacting oxidative stress, such as environmental conditions, individual characteristics and behaviours, may act as potential predictors, cofounders, covariates, etc; sometimes they are detectable and measurable (e.g. body composition, age, smoking habits, and physical activity); otherwise, they show an even greater complexity (e.g. environment). Specifically, this PhD project aimed to investigate oxidative stress biomarker fluctuations in different populations. The research project has been structured in order to separate, as feasible, different ages. Then, we focused alternatively the attention on different influencing factors: individual (body composition: obesity) and collective (environmental exposures: xenobiotics and green spaces). The final goal was to analyse both individual and collective "exposures" taking into account also confounders and covariates, such as tobacco smoking exposure and physical activity, both related to lifestyles and behaviours, thus of primary interest in prevention strategies. In particular, the exercise-induced oxidative stress investigation had been originally planned to link personal, behavioural and environmental domains altogether. However, the COVID-19 Pandemic broke out during the third year of the project hampering any follow-up or the beginning of new study lines. Therefore, a "satellite" activity has been set up to pursue slightly different objectives on the same topic. With this purpose, a systematic review and meta-analysis have been carried out, providing a theoretical basis for future investigations on physical activity and oxidative stress in adults.

3.2. Specific objectives

3.2.1. Study line I: individual and environmental factors in children

All the phases of the Study line I were handled by the PhD candidate supported by her tutor and research team and in collaboration with the Department of Agricultural, Forest and Food Sciences (University of Turin, Turin, Italy) and ISGlobal (Instituto de Salud Global, Barcelona, Spain), where the candidate has spent six months as pre-doctoral fellow.

First, the sampling procedures, which followed the recruitment and enrolment of all participants. Second, the handling of the biological samples and their laboratory analysis. Third, the characterisation of additional exposures (e.g. BMI reference standards identification; geospatial analyses. Further details in the "Material and Methods" section). Finally, the statistical analyses, the conceptualisation and the preparation of the manuscripts. The study line I has been implemented:

- To assess the effects of obesity, defined by BMI, on systemic oxidative stress in school-aged children living in Asti, accounting for several confounding factors. Moreover, to assess which BMI reference standard is more accurate in classifying obese children for the association with oxidative stress.
  Manuscript 1: **Squillacioti G**, Bellisario V, Grignani E, et al. "*The Asti study: The induction of oxidative stress in a population of children according to their body composition and passive tobacco smoking exposure*". Int J Environ Res Public Health. **2019**;16(3). doi:10.3390/ijerph16030490
- To assess the effects of urban green spaces on systemic oxidative stress in school-aged children living in Asti, accounting for the mediation role of physical activity.

Manuscript 2: "*Residential and school greenness exposure and oxidative stress in children. A cross-sectional study*". **Squillacioti G**, Carsin AE, Bellisario V, Dadvand P, Bono R, Garcia-Aymerich J. To be submitted.

3.2.2. Study line II: individual and environmental factors in adolescents

The PhD candidate collaborated with her tutor and research team (i) performing and managing the spatial analyses; (ii) carrying out some of the biological and statistical data analyses; (iii) collaborating in the conceptualisation and/or preparation of the manuscripts. The study line II has been implemented:

- To define a map of oxidative stress risk based on the association between urban green spaces and systemic oxidative stress in adolescents living in Turin.
  Manuscript 3: "*Geomatics and Epidemiology: Associating Oxidative Stress and Greenness in Urban Areas"* De Petris S*, **Squillacioti G***, Bono R, Borgogno Mondino E. Under review by Environmental Research journal.
- To assess the effects of bisphenol A on systemic oxidative stress, in both school-aged children and adolescents living in Chivasso (TO) accounting for age-dependent and tobacco smoking exposure differences.
  Manuscript 4: Bono R, Bellisario V, Tassinari R, **Squillacioti G**, Manetta T, Bugiani M, Migliore E, Piccioni P "Bisphenol A, Tobacco Smoke, and Age as Predictors of Oxidative Stress in Children and Adolescents". Int J Environ Res Public Health 2019; 16(11):2025 doi:10.3390/ijerph16112025

3.2.3. Study line III: Systematic Review and meta-analysis on exercise-induced oxidative stress in adults

As previously mentioned, the original plan was to jointly investigate the oxidative stress induction taking into account environmental features, individual characteristics and physical activity (considered as an example of lifestyle) during the last year of this doctoral project. However, due to the COVID-19 pandemic, the PhD candidate has been forced to change her schedule, carrying out a systematic review and meta-analysis on a similar topic:

- To assess the most appropriate exercise-induced biomarker in non-invasive media (i.e. urine and saliva) for future application in physical activity and oxidative stress association.
  Manuscript 5 – Systematic Review and meta-analysis: "*Non-invasive biomarkers to quantify exercise-induced oxidative stress in saliva and urine. A systematic review and meta-analysis"* Squillacioti G, Colombi N, Guglieri F, Ghelli F, Gardois P, Berchialla P, Bono R. To be submitted.

4. MATERIALS AND METHODS

This section reports specific subsections (4.1-4.4) describing the extended methods and materials used to carry out the observational studies which are part of the study line I and II. By contrast, the study line III will be described separately in section 4.5, as it refers to the systematic review and meta-analysis (Study line III).

4.1. Study designs, sample populations and Ethic approvals

All the studies presented in this doctoral thesis were performed in northern Italy, in the Piedmont region that is located in the Po valley area (**Figure 2**). The Po Valley (470,000 km^2) is one of the largest European plains, relatively homogeneous in terms of lifestyle, social and working conditions of the dwellers living and commuting in this territory. In ecological terms, its climate is continental and, due to frequent thermal inversion episodes, vertical and horizontal air exchanges are more difficult compared to other areas of Europe, thus the air quality is poor (Diémoz et al. 2019). All the aforementioned factors, together with the presence of the Alps, can lead to the stagnation of the air masses that reduces the pollutants dilution. Based on these environmental characteristics, this specific study area deserves an in-depth investigation, especially from a Public Health standpoint.

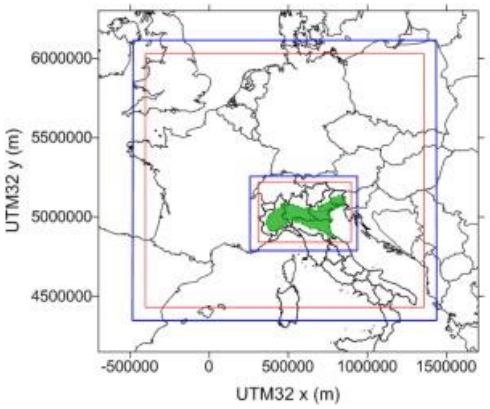


Figure 2 *Po Valley location (Finardi 2014)*

4.1.1. Sample population of children living in Asti (Study line I)

The study line I includes a cross-sectional study that was carried out involving a sample of 330 school-aged children (8-11 years), who were recruited from five primary schools located in the municipality of Asti. Asti is a medium town of 75,528 inhabitants (499 inhabitants/km^2) (ISTAT 2019), located 123 meters above sea level (a.s.l.) in the Piedmont region. All subjects participated as volunteers and they were enrolled according to the following inclusion criteria: only healthy children ranging from 8 to 11 years by March 2017 and resident in the selected area. Since the subjects were underage, their parents or legal tutors were asked to sign an informed consent allowing their participation.

This study was approved by the local Ethic Committee "*Comitato Etico Internazionale Azienda Ospedaliero-universitaria Luigi Gonzaga*" (no. 0005540, II, cat. 02, Cl. 01).

4.1.2. Sample populations of adolescents living in Torino and Chivasso (Study line II)

Two studies and sample populations were considered in the study line II:

First, a sample population of adolescents who were part of a larger research project funded by the Piedmont Regional Council focusing on the effects of environmental pollution in schoolers in 2002-2010. The analyses included in this PhD thesis refer to 205 healthy adolescents (10–13 years) enrolled from secondary schools located in Turin. Turin is a city of 867,620 inhabitants (6,700 inhabitants/km^2) (ISTAT 2020), located 239 a.s.l., and is the capital of the Piedmont Region. All subjects participated as volunteers and, because they were minors, their parents (or legal tutors) signed a written informed consent to allow participation.

The study protocol was approved by the local Ethics Committee "*Comitato Etico Internazionale Azienda Ospedaliero-universitaria Luigi Gonzaga*"" (no. 826/13/08).

Another sample population, including both children and adolescents (7-19 years), who were recruited from three primary and secondary schools located in the municipality of Chivasso was

analysed. Chivasso is a small town of 26,908 inhabitants (525 inhabitants/km^2) (ISTAT 2018), located 180 m a.s.l. in the Piedmont Region. All subjects participated as volunteers and they were enrolled according to the following inclusion criteria: only healthy subjects ranging from 7 to 19 years of age by March 2015, and resident in the selected area. Since the subjects were underage, their parents signed a written informed consent. This study received the approval of the local Ethics committee of "*Comitato Etico Internazionale Azienda Ospedaliero-universitaria Luigi Gonzaga*" (no. 27/2015).

4.2. Exposure variables

Depending on the study line (I and II), several exposure variables have been considered. The following paragraphs report the extended methods, which have been used to define and measure them.

4.2.1. BMI and body composition measurements (Study line I)

Measurements of body composition were performed using an impentiometric scale (FitScanBC-545F Tanita®), which adopts advanced BIA technology. Each subject underwent measurements wearing light clothes and without shoes and socks. For children (aged 7–17), the scale only displays three parameters, namely body weight (kg), BMI (Kg/m^2), and Body Fat (%) (BF). To categorise subjects according to their body weight, BMI was used as proxy of body composition. It was categorised according to three different classification systems proposed by the Centers for Disease Control and Prevention (CDC) (Kuczmarski et al. 2002), the International Obesity Task Force (IOTF) (Cole et al. 2000, 2007), and the WHO (de Onis et al. 2007), respectively. All cut-off values were sex and age-adjusted. UW, NW, OW, and OB categories were defined as (i) extrapolation of the adult BMI cut-off points according to the IOTF standard, (ii) based on centiles, in the case of the CDC reference, and (iii) according to standard deviations from the mean values of the WHO reference standard.

4.2.2. Individual exposure to greenness: NDVI (Study line I)

The Normalised Difference Vegetation Index (NDVI) has been used to quantify the individual exposure to greenness, by calculating the amount of vegetated biomass surrounding each participant's home. The NDVI metric has been calculated within circular buffers surrounding the subject's home residence. Hence, as a preliminary phase, all residence addresses were geocoded using a specific plugin with a declared positioning accuracy lower than 25m (Cetl et al. 2018). All spatial analyses were operated by SAGA GIS vs.7.1 and QGIS vs. 3.4.12. A multispectral and cloud-free satellite image (spatial resolution 10 x 10 m), was acquired the same year of the sampling campaign by Sentinel-2 (S2) satellite, downloaded from Theia CNSE website. The assumption was that June-July months are the period of maximum vegetation phenology expression in the study area (De Petris et al. 2019; Zhou et al. 2016), thus acquiring a summer satellite image allows quantifying the maximum exposure to vegetation. The NDVI is a widely used index of vegetated biomass that ranges from –1 to 1. This index is calculated exploiting the leaves chlorophyll capacity of mostly reflecting the near-infrared band (NIR) (0.7–1.1 µm) and strongly absorbing the visible light (0.4–0.7 µm) (formula 1). For each participant the following variables were derived: (i) NDVI average and (ii) vegetated portion. The vegetated portion was the ratio between the vegetated area and the whole buffer area (the vegetated area was calculated by masking out non-vegetated pixels, NDVI < 0.40). To derive a multisite exposure, the same variables around each school which accounted for weighed greenness exposure by time spent at school (8h/day) and at home (16h/day), were derived: (iii) weighted NDVI average and (iv) weighted vegetated portion.

$$NDVI = \frac{(NIR - RED)}{(NIR + RED)} \quad (1)$$

NIR: Near-infrared reflectance

RED: Red reflectance

4.2.3. Individual exposure to bisphenol A (Study line II)

All the urinary samples used for the bisphenol A quantification, have been collected using bisphenol A-free vessels and stored at -80 °C until analysis. Moreover, to avoid any accidental contaminations of the samples, laboratory glass material has been pre-treated with methanol and kept in methanol for 12 hours prior to being used. Each urinary sample (400 µL) was thawed and vortexed, then acetonitrile (700 µL), ethyl acetate (750 µL), BPA-d16 used as internal standard (10 µL from a stock solution with a concentration of 1 ng/µL) were added. To facilitate the liquid-liquid extraction, samples were vortexed for 3 minutes, then centrifuged at 4000 rpm for 15 minutes. The resulting supernatant was collected and evaporated to dryness under a gentle stream of nitrogen. In collaboration with the *"Department of Molecular Biotechnology and Health Sciences, Unit of Mass Spectrometry, of the University of Turin*", the bisphenol A was quantified as follows. The dried extract was dissolved with 125 µL of methanol/water (1:1 v/v) and analysed by High-Performance Liquid Chromatography coupled with Mass Spectrometry (HPLC—MS/MS) to quantify GlcA–BPA. The liquid chromatography was equipped with a low-pH resistant reverse phase column, Kinetex EVO C18 (2.6 µm, 150 x 3.0 mm). The binary solvent system was: (a) acidified ultrapure water with formic acid 0.1% v/v and (b) acetonitrile (HPLC ultrapure grade) acidified with formic acid 0.1% v/v. The chromatographic separation was carried out at constant flow rate (200 µL/min) and constant temperature (23 ± 1 °C). The injection volume was 20 µL, and the quantification was performed by using the internal standard method (BPA-d16). Quantitative analyses were carried out by tandem mass spectrometry with a 6330 Series Ion Trap LC-MS system equipped with an electrospray ionization source. The analytes were detected in negative mode. Procedural blank samples with ultrapure water in the place of urine were collected, extracted, and analysed by HPLC-MS/MS with the same sample protocol.

4.2.4. Individual exposure to greenness: SAVI and map of trees (Study line II)

The Soil Adjusted Vegetation Index (SAVI) was empirically derived from NDVI (formula 2), since in urban contexts soil background exerts a considerable influence on the average pixel spectrum, influencing the accuracy of exposure characterisation. SAVI has been used to quantify the individual exposure to greenness, by calculating the amount of vegetated biomass surrounding each participant's home (preliminary geocoded, as abovementioned for the NDVI calculations). All spatial analyses were operated by SAGA GIS vs.7.1 and QGIS vs. 3.4.12. Non-vegetated areas (buildings, streets etc.) were masked out by SAVI thresholding: pixel showing SAVI values < 0.45 were labelled as not-vegetated (Borgogno-Mondino et al. 2016). Different variables have been generated within a buffer (500-m radius) surrounding each participant's home: (i) masked SAVI mean value (mSAVI); (ii) the percentage of green cover (PGC) by comparing the vegetation fraction falling within each buffer with the whole area of the buffer; (iii) unitary SAVI (uSAVI) obtained by multiplying PGC and mSAVI to measure biomass density.

(2)

$$SAVI = \frac{1.5\ (NIR - RED)}{(NIR + RED + 0.5)}$$

Additionally, a map of trees *census*, updated at 2019 (nominal scale 1:1000), was provided by the Torino Geoportal website. The map of tress contains more than 160,000 trees divided in evergreen and broadleaf species and has been used to quantify the number of evergreen and broadleaf trees falling in the residential buffers. Hence, two more variables, accounting for the number of trees

falling in the 500-m-radius buffers, were calculated, namely (i) Number of Evergreen Trees (NET) and (ii) Number of Broadleaf Trees (NBT).

4.2.5. Individual exposure to tobacco smoking: urinary cotinine (Study lines I and II)

Cotinine was quantified as a biomarker of tobacco smoking exposure as follows: 10 mL of urine were transferred into a glass tube, then 4.25 g of NaCl, 500 µL of NaOH (5M), and 10 µL of cotinine-d3 (internal standard) were added. To perform a double liquid-liquid extraction, 2 mL of trichloromethane ($CHCl_3$) were added. The sample was centrifuged for 10 minutes at 3000 rpm and the resulting organic phase was collected and transferred into a new glass tube where was evaporated to dryness under a gentle stream of nitrogen. The cotinine quantification has been carried out in collaboration with the *Istituti Clinici Scientifici Maugeri* (Pavia, Italy). The dry residue was reconstituted in 200 µL of $CHCl_3$ and transferred into a conical vial for the quantification by Gas Chromatography-Mass Spectrometry determination (GC-MS). The GC-MS analysis was performed using an Agilent Technologies 6890 GC, interfaced to a 5973 MSD Inert Agilent mass spectrometer. A Gerstel CIS4 PTV injection system utilized an initial temperature of 50 °C followed by heating at 10 °C/s; with a final temperature of 300 °C, held for 10 minutes. The injection volume was 1 µL in the split mode. The capillary column used was an HP-5MS of 30 m × 0.25 mm × 0.25 µm film thickness. The initial column temperature was 50 °C, increased at 15 °C/minute up to 300 °C. The carrier gas was ultrapure Helium (1.0 mL/minute). The transfer-line temperature was set at 280 °C. The MS operated in electron impact and SIM mode. The monitored m/z values for cotinine were: 98, 118, 176; while those for the internal standard were 101, 121, 179. The cotinine calibration curve was built by fortifying a blank urine pool of non-smoking subjects, to obtain a concentration ranging from 0.02 µg/mL to 2 µg/mL. The fortified urine was extracted for the samples. The Limit Of Detection (LOD) and the Limit Of Quantification (LOQ) were 0.01 µg/mL and 0.02 µg/mL, respectively. The Coefficient of Variations (CVs), calculated to test the repeatability, were below 5% for cotinine and the internal standard. Since urinary cotinine was measured in spot urine, it has been expressed as ng/mg of Creatinine (Crea) accounting for the excretion rate normalisation. Urinary Crea has been quantified by the Jaffé method (Bonsnes and Taussky 1945) in each urinary sample.

4.2.6. Standardised questionnaire: other covariates and confounders (Study lines I and II)

A standardised questionnaire ("SIDRIA" questionnaire (Renzoni 1997)) was self-administered to the parents or legal tutors of each participant. The questionnaire covered several domains to give an insight into the potential confounder variables and covariates such as personal data, residence address, house feature, respiratory symptoms and allergies, parental education level, parental nationality, diet habits, sedentary behaviour and physical activity. In particular, **physical activity** was assessed by asking the question "*How many days per week does your child play sport or spend time being active/doing physical activity for at least 60 minutes (extra-scholastic)?*" The answer (days) underwent re-codification into 4 groups: ≤1 day/2/3/≥ 4 days. **Parental education** level was assessed by asking the higher qualification of each parent separately and the answer (qualification) underwent re-codification into 2 groups: low education level (up to lower secondary school) and high education level (upper secondary school and beyond).

4.3. Outcome variables

In the study lines I and II systemic oxidative stress has been measured by the quantification of urinary 15-F2t IsoP biomarker, as the main outcome variable. As previously mentioned for urinary cotinine, also 15-F2t IsoP has been expressed as ng/mg Crea.

4.3.1. Oxidative stress: 15-F2t IsoP quantification (Study lines I and II)

All the subjects involved in this PhD project provided a sample of spot urine for the biological analyses. All samples have been handled, aliquoted, stored at -80°C, and analysed in the Laboratory of Environmental Hygiene – Department of Public Health and Pediatrics (University of Turin).

Urinary 15-F2t IsoP has been measured as oxidative stress biomarker by ELISA technique performed by a specific microplate kit assay (Oxford, MI, USA), according to manufacturer's instructions. The declared limit of detection is 0.2 ng/mL and cross-reactivity can potentially occur for other isoprostanes such as prostaglandin E2, prostaglandin D2, and arachidonic acid (0.01%), 9α,11βprostaglandin F2α (4.1%), 13,14 dihydro-15-keto-PGF2α (3.0%).

All urinary samples were pre-treated with a preliminary incubation with β-glucuronidase since over 50% of 15-F2t-IsoP is glucuronic-conjugated in human urine (Romanazzi et al. 2013). Additionally, a sample dilution with the enhanced dilution buffer was adopted to achieve better accuracy. The enhanced dilution buffer prevents from samples purification, because it eliminates interferences due to non-specific binding. The procedure principle is that the samples (or standards) compete with the 15-F2t IsoP that is conjugated to the horseradish peroxidase for binding to a polyclonal antibody specific for 15-F2t IsoP coated on the microplate. The activity of horseradish peroxidase results in a colour development when the substrate is added, and the colour intensity is directly proportional to the amount of 15-F2t IsoP bounded to the horseradish peroxidase and inversely proportional to the amount of the unconjugated 15-F2t IsoP, thus the quantity of the samples (or standards).

Briefly, the aliquoted urinary samples are thawed from -80°C and 100 µL of them are transferred into clean Eppendorf tubes for the β-glucuronidase (5 µL) pre-treatment, thus vortexed and incubated at 37°C for two hours. Samples are diluted 1:4 with the enhanced dilution buffer. An eight-point standard curve (B_0-S_7) is built by performing sequential dilutions starting from the 15-F2t-IsoP stock solution diluted using the enhanced dilution buffer. Standards or diluted urinary samples (100 µL) are added to each microplate well (**figure 3**), then the diluted 15-F2t-IsoP conjugate (100 µL) is added to each well (omitting the reagent blank cell where 100 µL of enhanced dilution buffer are added instead). The microplate is incubated for two hours at room temperature. Then, the content of each well is removed by inversion and the microplate is wiped. Three microplate washings are performed adding 300 µL of wash buffer to each well. The TMB Substrate (200 µL) is added to each well and another incubation is performed (20-40 minutes at room temperature, until an appreciable blue hue is observed for B_0). To stop the reaction, 50 µL of 3M sulfuric acid are added to each well, whose colour turns from blue to yellow. The microplate is read in absorbance (450 nm) using the Tecan Infinite ® 200 PRO spectrophotometer.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_7	S_7	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_6	S_6	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_5	S_5	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_4	S_4	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_3	S_3	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_2	S_2	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_1	S_1	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	B_0	B_0	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	RB	RB

Figure 3 *ELISA microplate scheme showing standards (B_0-S_7) and urinary samples (U_n) positions*

4.4. Statistical analyses

Irrespective of the study line (I and II), all descriptive analyses were performed and presented as follows: continuous variables were presented as mean (Standard Deviation, SD) or, if the distribution was not normal, median (Interquartile Range, IQR). Categorical variables were expressed as absolute and/or relative frequency (number of cases; differences between groups were tested using non-parametric Mann-Whitney U-test and X^2 test. Shapiro-Wilk test,

distributional diagnostic plots and descriptive statistics were used to evaluate the normality, skewness and variance stability of the distributions; based on the specific analyses, the variable of interest was transformed (log-transformation and Box-Cox transformation). Correlations between different exposure variables and oxidative stress were tested using parametric and non-parametric correlation tests (Pearson and Spearman's tests). By contrast, since the objectives were different among the study lines (I and II), diverse statistical methods were used to perform the main analyses, depending on the study line itself. Further details on the specific statistical methods used to test the main hypotheses are reported in the following sub-paragraphs, grouped according to the study line.

4.4.1. The associations between obesity, greenness and oxidative stress in children (Study line I)

To assess the association between obesity (categorised BMI) and oxidative stress (15-F2t-IsoP), a Generalised Linear Model (GLM) with a log-link function and a Gaussian distribution was used, adjusting for cotinine quartiles, physical activity, gender, age and body fat percentage. Three GLMs were used to compare the association of oxidative stress and differently-categorised BMIs (each categorisation was based on IOTF, CDC and WHO reference standards, respectively).

To assess the association between greenness (NDVI) and oxidative stress (log-transformed 15-F2t-IsoP) a Linear Mixed Model (LMM), including age as covariate and schools as a random intercept accounting for the potential heterogeneity of sampling, was used. The parsimonious model was age-adjusted and was fitted by the Restricted Maximum Likelihood (REML) estimation. The goodness of fit was checked by verifying the normality of the residuals. Additionally, physical activity was added to the models as covariate to assess if it was a potential mediator in the association between greenness and oxidative stress (Baron and Kenny 1986). As sensitivity analyses, further variables (BMI, sex, cotinine, and parental education) were included in the LLMs, accounting for potential residual confounding. The main analysis was repeated using different greenness buffer sizes (100 m, 250 m, 300 m and 1000 m radii, one at a time). Further, a school-stratified analysis was performed and its estimates were combined by meta-analytical method of the inverse variance, DerSimonian-Laird estimator for Tau^2 and Jackson method for confidence intervals.

4.4.2. Spatialising the greenness and oxidative stress association and testing the association between bisphenol A and oxidative stress in adolescents (Study line II)

To spatialise the association between greenness variables (SAVI, percentage of vegetation and number of evergreen/broadleaf trees) and oxidative stress (15-F2t-IsoP), several Linear Regression Models (LRMs) were used. Preliminarily, the oxidative stress distribution was checked for outliers; then two-ways LRMs were performed for testing the association between 15-F2t-IsoP and SAVI, percentage of vegetation and number of evergreen/broadleaf trees, respectively. Since the abovementioned models showed a very poor association, an envelope function of the variable of interest was calculated by dividing distributions into 20 equiprobable classes. Then, the envelope function of the oxidative stress distribution (15-F2t-IsoP -Ev) was tested versus the vegetation variables, using LMs. Additionally, the Warton method (Warton et al. 2006) was used to evaluate which variable between NET and NBT, could have affected more oxidative stress.

To assess the association between bisphenol A (GlcA–BPA transformed by Box-Cox) and oxidative stress (15-F2t-IsoP transformed by Box-Cox), a Piecewise Linear Regression was used, adjusting for cotinine (log-transformed), BMI, gender and age classes. The prelaminar inspection of the two-way plot of oxidative stress (log-transformed 15F2t-IsoP) *versus* bisphenol A (log-transformed GlcA–BPA), helped in detecting a non-linear relationship, which suggested the existence of a threshold value of bisphenol A. The Piecewise Linear Regression presupposes that (i) two straight lines, with

different slopes, best fit the effect of the predictive variables on oxidative stress and (ii) there exists a breakpoint value of the independent variable at which the slope changes.

4.5. Systematic Review and met-analysis (Study line III)

As previously mentioned, a systematic review and meta-analysis were carried out speculating on which non-invasive biomarkers of oxidative stress could be used in investigating exercise-induced oxidative stress in adults. This paragraph reports the whole process by which the systematic review was performed and the statistical methods used in the meta-analysis.

4.5.1. The formulation of the research question

First and foremost, a detailed, clear, and specific research question has been formulated. The research question was based on practical field research needs, derived from the intention of investigating oxidative stress response due to physical activity, thus exercise-induced oxidative stress biomarkers. To support the epidemiologic approach, the field has been narrowed to non-invasive biomarkers only, those measured in saliva and urine. Moreover, since the original plan of this doctoral project was to analyse also adults, the research question has been restricted to the adult population. Preliminarily, a scoping literature review was performed to (i) avoid topic duplication prior to starting the effort of a systematic review (checking on-line platforms such as PROSPERO and scientific databases) and (ii) to better define the research question, according to the P.I.C.O. framework (P = Patient, Problem or Population; I = Intervention; C = Comparison, control or comparator; O = Outcomes).

4.5.2. (*A priori*) Protocol drafting and registration

A systematic review protocol was drafted *a priori*, to rigorously perform the systematic review. The final version of the protocol was submitted to the PROSPERO platform (The University of York, United Kingdom). PROSPERO is officially defined as "*an international database of prospectively registered systematic reviews in health and social care, welfare, Public Health, education, crime, justice, and international development, where there is a health-related outcome*" (https://www.crd.york.ac.uk/prospero/#aboutpage). The registration is important because it (i) provides transparency in the review process; (ii) helps counter the publication bias; (iii) helps to prevent biases by tracking any differences between the methods or outcomes reported in the published review and those planned in the registered protocol; (iv) helps improve the general quality of the systematic review and (v) allows identifying whether there are any reviews already underway addressing the same topic, thus avoiding unintended, time-consuming duplications. During this step, the eligibility criteria, for including or excluding searched articles in the systematic review, were clearly defined. **Table 2** reports the exclusion criteria.

Exclusion criteria (applied in to screen titles, abstracts and full-texts)
No full-text available
Animal/ in vitro studies; ecological/time-series designs
Oxidative stress biomarkers are measured in other specimens than urine and/or saliva
Non-objectively assessed physical activity (e.g. questionnaire)
Physical activity performed in extreme conditions (e.g. high altitude)
Participants are taking antioxidant supplementation
Participants are aged less than 18 years
Articles are written in other languages than English or Italian
Articles are not primary studies (e.g. reviews)
Articles are case studies, conference abstracts or editorials

Table 2 *The exclusion criteria used to screen abstracts and full-texts in the systematic review*

4.5.3. Search string building, validation and launch

The search strategy was defined using a specific search string, built collaborating with the "*Biblioteca Federata di Medicina Ferdinando Rossi"* of the University of Turin. Firstly, three keywords/concepts and their Thesaurus-based synonyms were linked using the Boolean terms (AND/OR). Secondly, the prototypal search string was validated launching it in PubMed and checking if it was able to find several articles, which were previously identified as "gold standard" (essential to answer the research question). Finally, the validated search string (**Annex II**) was launched on the 28th of April 2020, searching three electronic databases, namely PubMed, EMBASE and Cochrane CENTRAL.

4.5.4. Results cleaning and selection: abstracts and full-text screenings

Firstly, all the duplicate records were removed using the deduplication tool of the selected reference management software (Mendeley Ltd, vs. 1.19.5). Secondly, titles and abstracts were independently screened by the PhD candidate and another reviewer from the research team, by applying the eligibility criteria previously defined. If consensus could not be reached, a third reviewer arbitrated. All the procedure steps (4.5.4—4.5.6) were performed by the same reviewers independently. Thirdly, the included abstracts were searched for full-texts; then each full-text underwent the same screening procedures adopted for titles and abstracts.

4.5.5. Data extraction from included full-texts

A specific data extraction table was built using an excel spread-sheet, which was piloted prior to starting the actual data extraction phase, by extracting a small number of data. Data extraction table included several variables to be extracted, such as article ID, author, year of publication, journal, title, funding, conflict of interest, study design, subjects 'characteristics (sample size, age, sex, training/pathological/socio-economic *status*, etc.), intervention's characteristics (types of physical activity, duration, intensity, etc.) and biomarkers 'characteristics (name, biological matrix, analytical method, unit of measure, etc.). Moreover, the measure of the oxidative stress biomarkers was extracted at baseline and after physical activity, reporting the measures of central tendency associated to the measures of dispersion. The statistical tests and significance levels, used by the authors to test the effect of physical activity on oxidative stress, were also extracted and reported.

4.5.6. Quality assessment of included full-texts

Risk of bias (quality) assessment has been appraised by using validated instruments, specifically selected to this scope and study-design specific. Depending on the study design: i) the National Institute of Health (NIH) Quality Assessment Tools for observational, case-series, cross-sectional, and before-after studies, ii) the PEDro scale to assess the quality of randomised clinical trials, and iii) The Joanna Briggs Institute Critical Appraisal Checklist for controlled before-after studies. The aforementioned quality assessment tools define the quality of the articles by assigning a quality score (high score = less bias, higher quality). However, since they adopt different scales, results were expressed as a percentage and the quality score underwent re-coding based on the tertiles (1st tertile = poor quality; 2nd tertile = medium quality; 3rd tertile = high quality).

4.5.7. Meta-analysis: statistical methods

To estimate the pooled effect among different studies a random-effect meta-analytic model with the DerSimonian-Laird estimator (inverse variance method) has been used, then the Forest plot graphically summarised the results. The Hedges' g (Standardised Mean Difference SMD) statistic has been calculated as the summary measure and the Jackson method for confidence interval of Tau^2 and Tau was used. The heterogeneity was inspected by using the I^2 statistic. Moreover, to test the publication bias, both Eggers' test and Funnel plot have been carried out. Outliers have been identified as sensitivity analysis, and the influence analysis has been performed testing the influence of ach included article on the overall heterogeneity. Meta-regression models were built to check

the influence of (i) the quality of the included studies; (ii) types of physical activity and (iii) intensities of physical activity. All the aforementioned analyses have been performed using R software, version 4.0.2.

5. RESULTS

5.1. Body composition (BMI-based obesity) and oxidative stress in children (Study line I)

As previously mentioned, the epidemiologic sample of this study consisted of 330 children aged between 8 and 11 years, living in Asti. **Table 3** reports the description of the sample population sub-grouped by gender. Overall, there was a general homogeneity, underlined by none statistically significant differences between sexes by age, ethnicity and some of the measured anthropometric features (height, weight, and BMI). Conversely, the BF was higher in females compared to males.

Demographic characteristics		Fermale n=161 (48.8%)	Male n=169 (51.2%)	*P-value*	Overall 330 (100%)
Age (yrs.)	8	51 (31.7)	56 (33.1)	0.84	107 (32.4)
	9	58 (36.0)	53 (31.4)		111 (33.6)
	10+	52 (32.3)	60 (35.5)		112 (33.9)
Ethnicity (n)	Non-Caucasian[a] mothers	15 (9.3)	17 (10.6)	0.32	32 (9.7)
	Non-Caucasian[a] fathers	17 (10.1)	17 (10.1)	0.54	34 (10.3)
Height (cm)		138.4 (9.3)	138.9 (8.4)	0.57	138.4 (8.7)
Weight (kg)		36.5 (10.1)	36.8 (10.8)	0.78	36.3 (10.2)
BMI (kg/m^2)		19.1 ± 3.6	18.8 ± 3.6	0.28	18.8 ± 0.2
Body Fat (%)		26.9 ± 6.2	24.3 ± 6.6	< 0.0001	25.4 ± 6.5

Notes: Height, weight BMI, Body Fat %, are expressed as mean (SD)

[a] Non-Caucasian includes African, Asiatic, and Hispanic ethnicities.

Table 3 *Demographic characteristics of the sample population (n = 330). Study line I*

Figure 4 depicts the categorisations of children's body *habitus* based on three selected international BMI refence standards: IOFT, WHO and CDC. Each BMI reference standard categorised the children into four principal sub-groups: UW, NW, OW, and OB. Since, the UW group was less than 5% of the whole sample in each BMI standard, it has been considered together with the NW category. The prevalence of obesity varied using different BMI reference standards. In particular, WHO reported the highest numerousness of obese children (33.9%), CDC categorised almost equally OW and OB (22.4% and 21.2%, respectively), and IOTF showed the lowest prevalence of obesity (13.6%). Although the distribution of 15-F2t-IsoP did not change significantly over UW+NW, OW, and OB categories, in the IOTF groups there was a statistical tendency of 15-F2t-IsoP increase from UW+NW to OB category (p = 0.091) being the IOTF means(SD) equal to 4.5 (3.8), 4.9 (3.9), and 5.7 (4.7) ng/mg Crea in UW+NW, OW, and OB, respectively. According to the CDC categorisation, 15-F2t-IsoP was 4.3 (3.8), 4.8 (3.4), and 5.3 (4.7) ng/mg Crea in UW+NW, OW, and OB children, respectively (p = 0.356). The WHO categories of UW+NW, OW, and OB corresponded to 4.4 (3.9), 5.0 (3.3), and 5.1 (4.3) ng/mg Crea of 15-F2t-IsoP, respectively (p = 0.216).

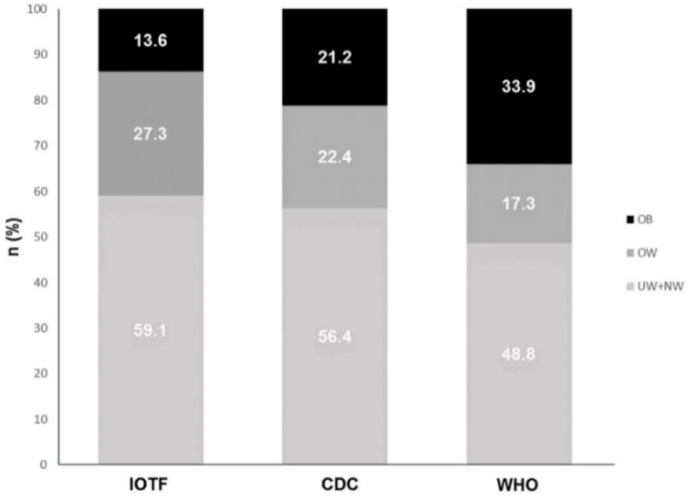


Figure 4 *Obese (OB), overweight (OW) under-weight (UW), and normal weight (NW) children according to the three selected BMI reference standards*

Table 4 reports the GLMs used to test the association between oxidative stress (15-F2t-IsoP) and obesity (categorised BMI). Among the three selected international BMI reference standards, only OB children categorised by the IOTF standard had significantly higher oxidative stress levels compared to NW children (1.56 95% C.I. from 1.07 to 2.27; $p = 0.020$). Additionally, tobacco smoking exposure was also associated to oxidative stress induction. In particular, children laying in the 4th and in the 3rd quartiles of urinary cotinine showed significantly higher levels of 15-F2t-IsoP ($p < 0.001$ and $p = 0.020$, respectively) compared to those detected in the 1st quartile.

		IOTF		CDC		WHO	
Oxidative stress		Exp(β) (95% C.I.)	*p*	Exp(β) (95% C.I.)	*p*	Exp(β) (95% C.I.)	*p*
BMI (categories)	UW+NW	*Ref.*		*Ref.*		*Ref.*	
	OW	1.22 (0.97; 1.56)	0.095	1.16 (0.91; 1.48)	0.224	1.22 (0.95; 1.58)	0.113
	OB	1.56 (1.07; 2.27)	**0.020**	1.30 (0.93; 1.82)	0.126	1.28 (1.07; 2.27)	0.081
Cotinine (quartiles)	1			*Ref.*		*Ref.*	
	2	1.27 (0.93; 1.72)	0.130	1.28 (0.94; 1.75)	0.111	1.29 (0.95; 1.76)	0.100
	3	1.45 (1.06; 1.97)	**0.020**	1.47 (1.07; 2.01)	**0.016**	1.45 (1.06; 1.98)	**0.020**
	4	2.04 (1.55; 2.69)	**<0.0001**	2.08 (1.57; 2.75)	**<0.0001**	2.12 (1.60; 2.80)	**<0.0001**
Physical activity (days/week)	0-1	*Ref.*		*Ref.*		*Ref.*	
	2-4	1.00 (0.83; 1.23)	0.944	1.02 (0.83; 1.24)	0.872	1.03 (0.85; 1.26)	0.720
	≥5	1.14 (0.81; (1.61)	0.440	1.16 (0.83; 1.63)	0.392	1.18 (0.84; 1.67)	0.337
Sex	Females	*Ref.*		*Ref.*		*Ref.*	
	Males	1.09 (0.92; 1.31)	0.297	1.09 (0.91; 1.32)	0.350	1.11 (0.93; 1.33)	0.249
Age (yrs.)		1.06 (0.96; 1.15)	0.210	1.05 (0.96; 1.15)	0.259	1.06 (0.97; 1.15)	0.234
Body fat (%)		1.00 (0.97; 1.01)	0.110	0.99 (0.97; 1.01)	0.319	0.99 (0.97; 1.01)	0.316

Table 4 *The Generalised Linear Models used to test the association between obesity and oxidative stress*

To test the robustness of the main results, additional models were built (**Table 5**). **Table 5** summarised four models that include a different set of variables, added step by step and re-coded when needed (i.e. cotinine quartiles). The association between oxidative stress and BMI-based obesity (assessed by the IOTF standard) was confirmed by these sensitivity analyses. Same analyses were carried out with the CDC and WHO BMI standards and they were consistent with the main results

Oxidative stress		Exp(β) (95% C.I.)	*p*	Exp(β) (95% C.I.)	*p*	Exp(β) (95% C.I.)	*p*	Exp(β) (95% C.I.)	*p*
Sex		1.02 (0.85; 1.22)	0.84	1.02 (0.85; 1.23)	0.82	1.01 (0.85; 1.21)	0.88	1.10 (0.92; 1.31)	0.29
Age (yrs.)		1.04 (0.95; 1.14)	0.37	1.05 (0.95; 1.15)	0.35	1.07 (0.99; 1.17)	0.10	1.06 (0.97; 1.15)	0.18
Body Fat (%)		0.99 (0.97; 1.01)	0.28	0.98 (0.96; 1.01)	0.17	0.99 (0.97; 1.00)	0.13	0.98 (0.96; 1.00)	0.10
BMI (IOTF)	UW+NW	*Ref.*		*Ref.*		*Ref.*		*Ref.*	
	OW	1.17 (0.92; 1.50)	0.20	1.23 (0.95; 1.58)	0.11	1.19 (0.95; 1.49)	0.14	1.23 (0.97; 1.57)	0.09
	OB	1.47 (1.02; 2.11)	**0.04**	1.66 (1.11; 2.48)	**0.01**	1.52 (1.11; 2.08)	**0.01**	1.56 (1.07; 2.26)	**0.02**
Physical activity (d/w)				1.01 (0.95; 1.08)	0.73	1.01 (0.95; 1.06)	0.78	1.01 (0.96; 1.07)	0.61
Cotinine (ng/mg Crea)						1.02 (1.02; 1.02)	**<0.001**		
Cotinine (quartiles)	1st							*Ref.*	
	2nd							1.27 (0.94; 1.72)	0.13
	3rd							1.45 (1.07; 1.98)	**0.02**
	4th							2.05 (1.56; 2.71)	**<0.001**

Table 5 *Additional models testing the association between obesity and oxidative stress*

5.2. Multi-site greenness exposure (NDVI), physical activity and oxidative stress in children (Study line I)

To assess the association between greenness (measured by NDVI) and oxidative stress (measured by 15-F2t-IsoP), a sub-sample of children (n = 323) has been included in the following analysis. Indeed, only 323 out of 330 provided the residential home address, which was essential for calculating the NDVI metric within circular buffers surrounding children's homes. The demographic characteristics of the sub-sample are summarised by **table 6**. Of note, since the fruition of urban green spaces might strongly depend on the socio-economic *status*, parental education has been reported hereinafter as a proxy of socio-economic condition. Moreover, physical activity has been analysed and reported with a different categorisation compared with the previously mentioned analysis, because it was suspected to affect the association between greenness and oxidative stress, thus deserved an in-depth analysis. Finally, descriptive analyses of the variables used to characterise the exposure to greenness (i.e. NDVI average, vegetated portion, time-weighted NDVI average and time-weighted vegetated portion calculated within 500-m buffers) have been reported too. Once again, the sample population showed a great homogeneity.

Characteristic			Female n=161 (50%)	Male n=162 (50%)	*P-value*	Total n=323 (100%)
Age (yrs.)			9.1 (0.9)	9.1 (1.0)	0.99	9.1 (1.0)
BMI (kg/m^2)			19.1 (3.6)	18.8 (3.5)	0.27	19.0 (3.5)
Cotinine (ng/mg Crea)			1.1 (2.2)	0.8 (1.6)	**0.02**	0.9 (1.9)
NDVI average			0.36 (0.17)	0.36 (0.17)	0.63	0.36 (0.17)
Vegetated portion			0.41 (0.23)	0.39 (0.23)	0.54	0.40 (0.23)
Time-weighted NDVI average			0.33 (0.12)	0.32 (0.12)	0.40	0.32 (0.12)
Time-weighted vegetated portion			0.37 (0.16)	0.35 (0.16)	0.19	0.36 (0.16)
Physical activity (days/week)		0-1	51 (32)	37 (23)	0.14	88 (27)
		2	55 (34)	47 (29)	0.43	102 (32)
		3	27 (17)	42 (26)	0.07	69 (21)
		≥ 4	28 (17)	36 (22)	0.32	64 (20)
Parental education	Mother	Up to lower secondary	66 (41)	66 (41)	>0.99	132 (41)
		Upper secondary and beyond	90 (56)	90 (56)	>0.99	180 (56)
	Father	Up to lower secondary	74 (46)	76 (47)	0.87	150 (46)
		Upper secondary and beyond	83 (52)	79 (49)	0.75	162 (50)

Notes: Age, BMI, and the greenness variables are expressed as mean (SD). Cotinine is expressed as median (IQR)

Table 6 *Demographics of the sample population (n = 323). Study line I*

To test the association between the greenness variables with oxidative stress, four multivariable LRMs were used (**table 7**). All greenness variables were inversely associated with oxidative stress (log-transformed 15-F2t-IsoP), attesting a decrease in oxidative stress for each 0.01 unit of increase in average NDVI (β: -0.39, 95% CI -0.85 to 0.05), in vegetated portion (β: -0.28, 95%CI -0.61 to 0.04), in time-weighted average NDVI (β: -0.63, 95%CI -1.27 to 0.02) and in time-weighted vegetated portion (β: -0.50, 95%CI -0.98 to -0.02), but the association was statistically significant only for time-weighted vegetated portion. An additional adjustment by physical activity to each of the four models slightly attenuated the magnitude of the associations between greenness and oxidative stress, suggesting that physical activity may mediate this association. Children reporting the lowest and highest frequencies of physical activity were those who had also the higher levels of oxidative stress (β: +0.19, 95%CI 0.04 to 0.43; β: +0.25, 95%CI 0.04 to 0.46) compared to the moderately-active children (**table 7, additionally adjusted models**).

Log (15-F2t IsoP)		Main models (age-adjusted only)		Additional adjustment (by physical activity)	
		Coeff. (95% C.I.)	*P-value*	Coeff. (95% C.I.)	*P-value*
NDVI average		-0.39 (-0.83; 0.05)	0.08	-0.31 (-0.76, 0.13)	0.16
Physical activity (days/week)	0-1			0.24 (0.04, 0.43)	**0.02**
	2			*Ref.*	
	3			0.16 (-0.04, 0.37)	0.12
	≥4			0.25 (0.04, 0.46)	**0.01**
vegetated portion		-0.28 (-0.61; 0.04)	0.09	-0.23 (-0.56, 0.10)	0.17
Physical activity (days/week)	0-1			0.24 (0.04, 0.43)	**0.02**
	2			*Ref.*	
	3			0.16 (-0.04, 0.37)	0.12
	≥4			0.25 (0.05, 0.47)	**0.02**
Time-weighted NDVI average		-0.63 (-1.27; 0.02)	0.06	-0.51 (-1.16, 0.13)	0.12
Physical activity (days/week)	0-1			0.23 (0.04, 0.43)	**0.02**
	2			*Ref.*	
	3			0.16 (-0.05, 0.37)	0.12
	≥4			0.25 (0.04, 0.46)	**0.02**
Time-weighted vegetated portion		-0.50 (-0.98; -0.02)	**0.04**	-0.42 (-0.90, 0.07)	0.09
Physical activity (days/week)	0-1			0.19 (0.04, 0.43)	**0.02**
	2			*Ref.*	
	3			0.15 (-0.05, 0.37)	0.12
	≥4			0.25 (0.04, 0.46)	**0.02**

Table 7 *Multivariable Linear Mixed Models testing the association between greenness and oxidative stress, only age-adjusted (left-hand part of the table) and further adjusted by physical activity (right-hand part of the table)*

All the aforementioned analyses referred to greenness variables calculated within 500-m residential buffers. However, to ascertain the robustness of the results, a set of sensitive analysis were carried out. Firstly, to test any potential residual confounding, the main analysis was further adjusted for BMI, sex, cotinine, and parental education. The set of confounders was established by checking for their relation with outcome/exposure variables, and by reviewing previous literature (**table 8**). Secondly, the main analysis was rerun by using different buffer sizes (100 m, 250 m, 300 m and 1000 m radii, one at a time) (**table 9**). Finally, we stratified our analysis by schools and combined those estimates using the meta-analytical method of the inverse variance, DerSimonian-Laird estimator for Tau2 and Jackson method for confidence intervals (**figure 5**).

Log (15-F2t IsoP)		NDVI average		Vegetated portion		Time-weighted NDVI average		Time-w. vegetated portion	
		Coeff. (95% C.I.)	*p*	Coeff. (95% C.I.)	*p*	Coeff. (95% C.I.)	*p*	Coeff. (95% C.I.)	*p*
Greenness variables		-0.47(-0.92, -0.01)	**0.04**	-0.33 (-0.67, 0.01)	**0.06**	-0.72 (-1.39, -0.05)	**0.04**	-0.57(-1.06, -0.07)	**0.03**
Age (yrs.)		0.08 (0.01, 0.17)	**0.04**	0.08 (0.01, 0.17)	**0.04**	0.09 (0.01, 0.17)	**0.04**	0.09 (0.01, 0.17)	**0.04**
Sex (1: male; 2: female)		-0.09 (-0.24, 0.06)	0.25	-0.09 (-0.24, 0.06)	0.25	-0.09 (-0.24, 0.06)	0.25	-0.09 (-0.24, 0.06)	0.25
BMI (kg/m^2)		0.01 (-0.01, 0.04)	0.28	0.01 (-0.01, 0.04)	0.28	0.01 (-0.01, 0.04)	0.28	0.01 (-0.01, 0.04)	0.28
Cotinine (ng/mg Crea)		0.01 (-0.00, 0.01)	0.09	0.01 (-0.00, 0.01)	0.11	0.01 (-0.00, 0.01)	0.10	0.01 (-0.00, 0.01)	0.10
Father education	Higher	*Ref.*		*Ref.*		*Ref.*		*Ref.*	
	Lower	-0.04 (-0.21, 0.13)	0.65	-0.04 (-0.21, 0.13)	0.67	-0.04 (-0.21, 0.13)	0.64	-0.04 (-0.21, 0.13)	0.66
Mother education	Higher	*Ref.*		*Ref.*		*Ref.*		*Ref.*	
	Lower	0.09 (-0.09, 0.27)	0.31	0.09 (-0.09, 0.27)	0.32	0.09 (-0.09, 0.27)	0.31	0.09 (-0.09, 0.27)	0.31

Table 8 *Multivariable Linear Mixed Models fully-adjusted to test the robustness of the association between greenness variables and oxidative stress*

Log (15-F2t IsoP)	Radius 100 m		Radius 250 m		Radius 300 m		Radius 1,000 m	
	Coeff. (95%CI)	*p*	Coeff. (95%CI)	*p*	Coeff. (95%CI)	*p*	Coeff. (95%CI)	*p*
NDVI average	-0.15 (-0.57, 0.28)	0.50	-0.28 (-0.71, 0.15)	0.20	-0.32 (-0.76, 0.11)	0.14	-0.52 (-0.99, -0.05)	0.03
vegetated portion	-0.10 (-0.30, 0.20)	0.52	-0.20 (-0.51, 0.12)	0.22	-0.29 (-0.54, 0.09)	0.13	-0.40 (-0.76, -0.03)	0.03
Time-weighted NDVI average	-0.27 (-0.90, 0.37)	0.41	-0.47 (-1.11, 0.17)	0.15	-0.56 (-1.18, 0.10)	0.09	-0.80 (-1.50, -0.09)	0.03
Time-weighted vegetated portion	-0.21 (-0.64, 0.23)	0.35	-0.38 (-0.83, 0.09)	0.10	-0.43 (-0.90, 0.03)	0.07	-0.62 (-1.17, -0.07)	0.03

Table 9 *Multivariable Linear Mixed Models age-adjusted to test the robustness of the association between greenness variables and oxidative stress by using differently-sized radii*

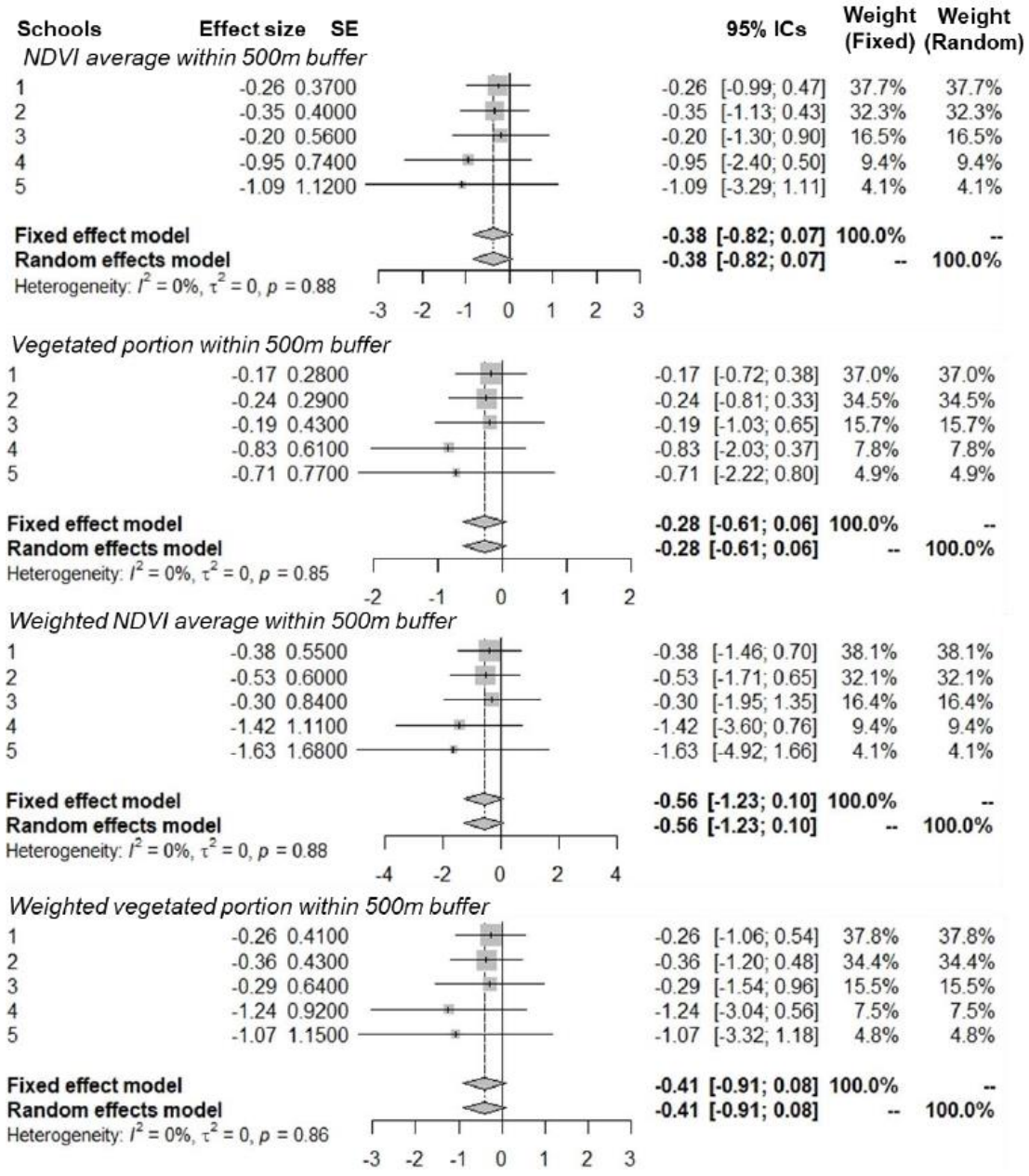


Figure 5 *Meta-analyses performed to test the possible bias due to the sampling location (schools)*

5.3. Greenness exposure (SAVI and trees) and oxidative stress in adolescents (Study line II)

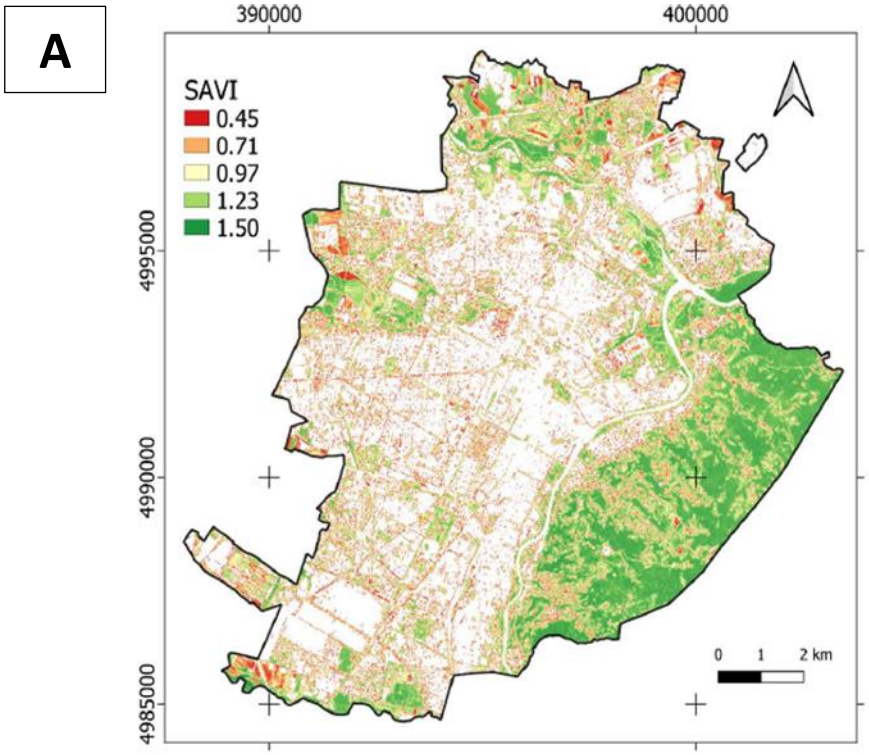
The epidemiologic sample of this study consisted of 205 adolescents aged between 8 and 11 years, living in Turin. **Table 10** summarised the descriptive analyses of the sample population that has been sub-grouped by gender. Considering the demographic characteristics, this sample population was homogeneous, since no statistically significant differences between sexes by age, height, weight, BMI were observed. Moreover, neither oxidative stress biomarker (15-F2t-IsoP) nor tobacco smoking exposure (cotinine) showed any differences between genders, and the same result has been observed for the greenness variables (mSAVI, uSAVI, NET, NBT, and PGC).

Characteristics	Female	Male	*P-value*	Overall
	n = 87 (42.4%)	n = 118 (57.6%)	0.04	n = 205 (100%)
Height (cm)	148.6 (8.4)	150.6 (9.6)	0.23	149.8 (9.1)
Weight (kg)	41.5 (9.7)	45.1 (12.0)	0.09	43.6 (11.2)
BMI (kg/m^2)	18.6 (3.2)	19.7 (3.7)	0.09	19.2 (3.6)
Age (yrs)	11.4 (0.7)	11.6 (0.9)	0.34	11.5 (0.8)
15-F2t-IsoP (ng/mg Crea)	9.9 (7.2)	10.8 (7.6)	0.58	10.5 (7.6)
Cotinine (ng/mg Crea)	0.6 (0.7)	0.6 (0.5)	0.70	0.6 (0.6)
mSAVI	0.85 (0.09)	0.86 (0.07)	0.26	0.86 (0.08)
uSAVI	0.33 (0.24)	0.30 (0.21)	0.10	0.31 (0.21)
NET	26 (70)	31 (60)	0.77	28 (64)
NBT	685 (508)	774 (461)	0.22	697 (461)
PGC	0.39 (0.24	0.36 (0.23)	0.10	0.34 (0.23)

Notes: Height, weight, BMI, age and the greenness variables are expressed as mean (SD).

Table 10 *Demographics of the sample population (n =205). Study line II*

As a preliminary analysis, three maps were generated, illustrating (i) mSAVI (**Figure 6A**) and (ii) tree *census* (**Figure 6B**) within the municipality of Turin, afterwards overlapped with the residential buffers (500-m of radius) surrounding each participant's home. Additionally, each residential buffer has been classified by different colours, according to 15-F2t-IsoP (**Figure 6C**).



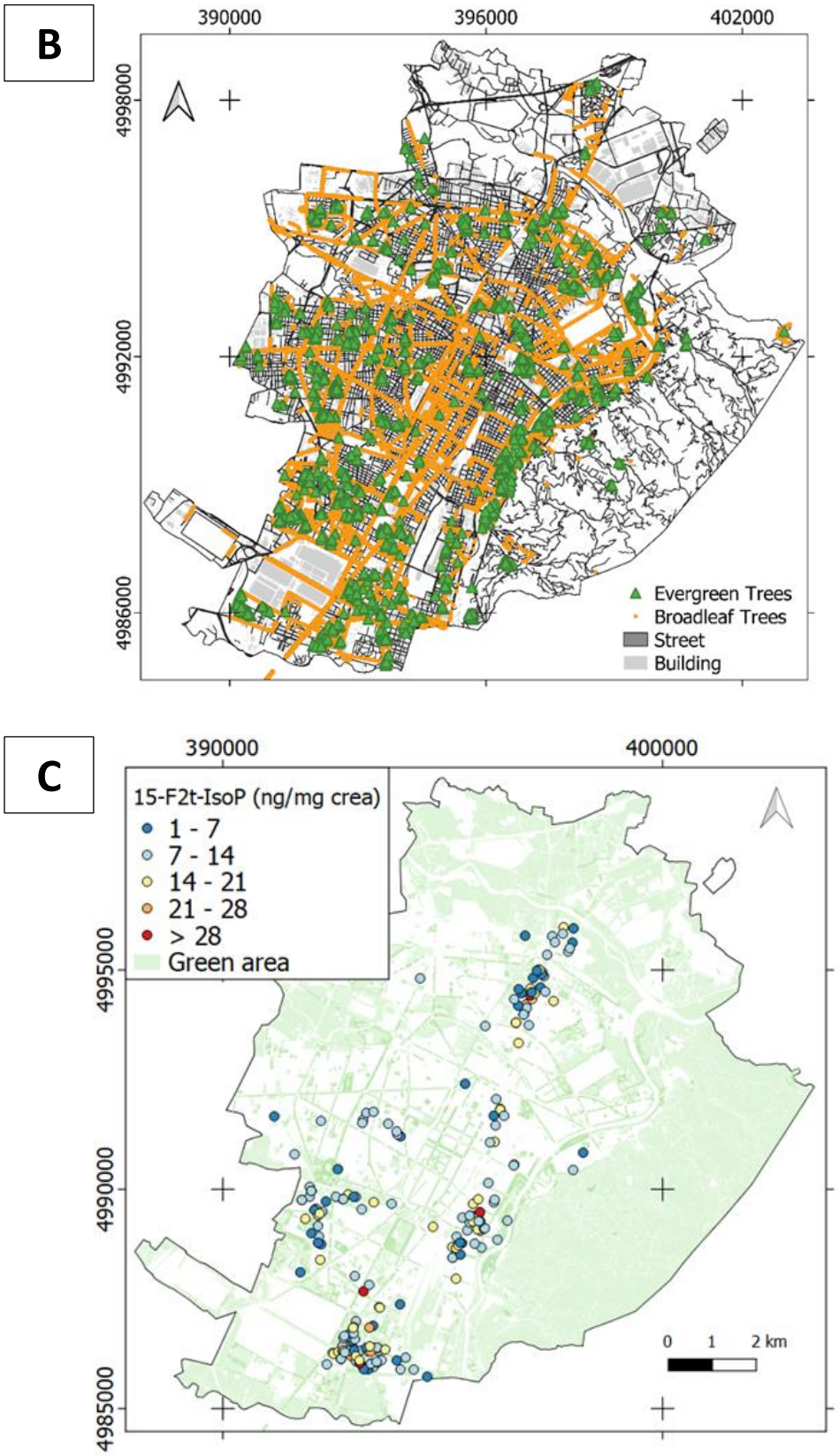


Figure 6 *Preliminary spatial analyses and related maps showing: greenness levels (A), evergreen and broadleaf trees (B) and 15-F2t-IsoP levels overlapped with the greenness map (C)*

Thus, Pearson's correlations and LRMs tested the associations between oxidative stress and uSAVI, PGC, NET and NBT, highlighting that oxidative stress and greenness variables were generally poorly associated (**table 11**).

Dependent variable	*r*	*P-value*	R^2	Slope	*p*(slope)	Slope (95% C.I.s)	Intercept
uSAVI	-0.045	0.520	0.002	-2.214	0.520	(-9.538, 4.685)	11.967
PGC	-0.046	0.519	0.002	-2.126	0.519	(-8.747, 4.819)	12.035
NBT	-0.028	0.691	0.001	-0.001	0.691	(-0.003, 0.002)	11.699
NET	-0.030	0.673	0.001	-0.005	0.673	(-0.025, 0.014)	11.523

Table 11 *Pearson's correlations and Linear Regression Models used to test the associations between oxidative stress and unitary SAVI, percentage of green cover, number of evergreen trees and number of broadleaf tress*

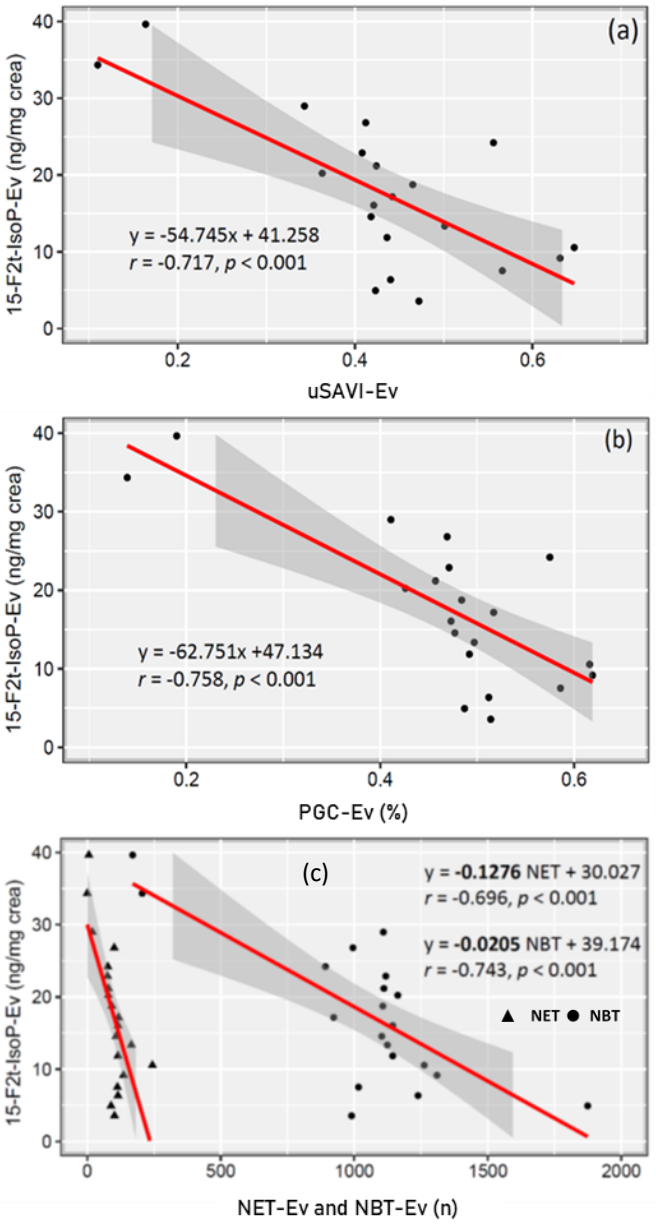


Figure 7 *Linear Regression Models testing the association between the envelope function of 15-F2t-IsoP and the unitary SAVI (a), the percentage of green cover (b) and the number of evergreen and broadleaf trees, respectively (c).*

Therefore, the envelope functions of 15-F2t-IsoP (15-F2t-IsoP-Ev), uSAVI (uSAVI-Ev), PGC (PGC-Ev), NBT (NBT-Ev), and NET (NET-Ev) have been estimated by using the maximum values extrapolated from the 20 equiprobable classes of each distribution.

The 15-F2t-IsoP-Ev metric was assumed to represent the distribution of the highest oxidative stress levels measured in the sample population, thus useful to speculate on the potential association between the extreme values of oxidative stress with greenness variables. A significant negative correlation was found between 15-F2t-IsoP-Ev and PGC-Ev ($r = -0.758$, $p < 0.001$) and between 15-F2t-IsoP-Ev and uSAVI-Ev ($r = -0.717$, $p < 0.001$). Hence, a higher vegetation cover seemed to significantly reduce oxidative stress. In particular, if vegetation cover is composed by trees (i.e. high uSAVI), oxidative stress tended to decrease (**Figure 7a and 7b**).

Moreover, the evergreen and broadleaf trees were tested separately (**Figure 7c**). In particular, NET determines a negative and steeper 15-F2t-IsoP-Ev reduction, six-time greater compared to NBT, suggesting that evergreen trees could determine a stronger positive effect on oxidative stress.

Finally, to spatialise the association between 15-F2t-IsoP-Ev and uSAVI-Ev, the related LRM was adopted. For this purpose, a 500 x 500 squared graticule (G) was generated assuming this size consistent with a walking mobility of 10–15 minutes (Wolch et al. 2014). For each cell of G, the uSAVI maximum value was computed from the previously generated SAVI masked map (**Figure 6A**). G was rasterised to generate uSAVI^{G}. A raster map (**Figure 8**) reporting the estimates of 15-F2t-IsoP-Ev (hereinafter called 15-F2t-IsoP-Ev^{G}) was finally computed by grid calculation tools, implementing the previously calibrated LRM (**Figure 7a**) and reporting the number of children actually living in Turin city. Of note, the map of 15-F2t-IsoP-Ev^{G} may be useful in locating urban areas with higher (dashed box) expected oxidative stress levels. Therefore, it may be considered a useful tool in simulating future urban scenarios where new green areas could be requalified or implemented.

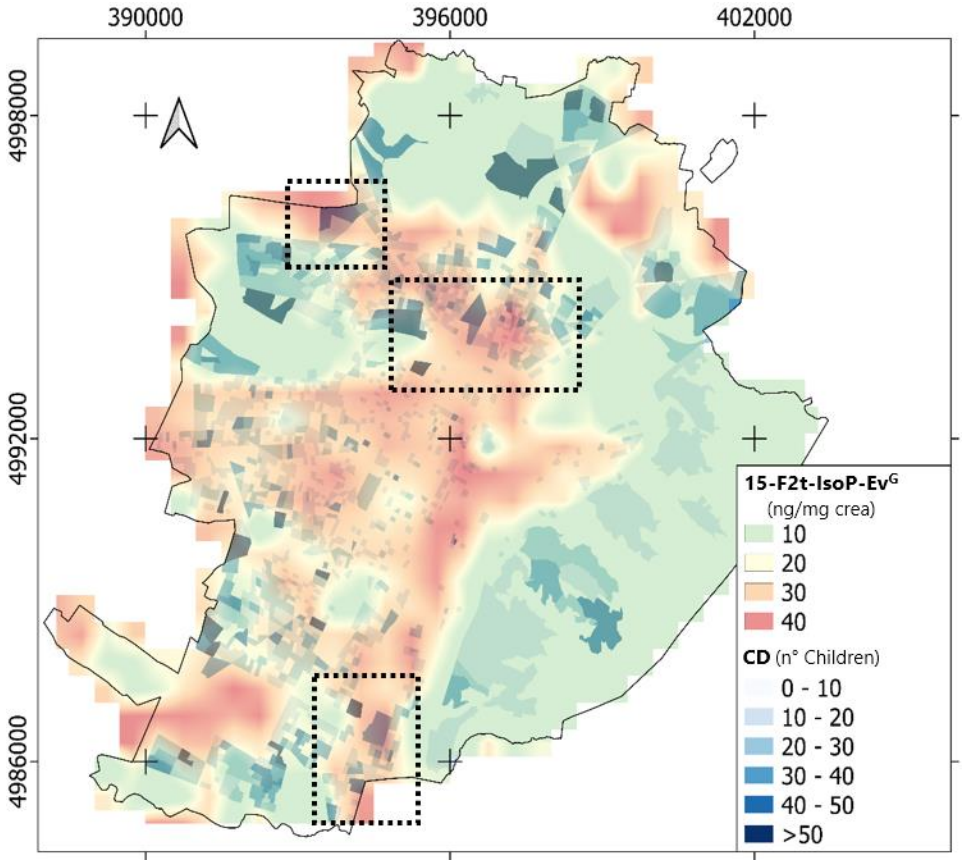


Figure 8 *Prototypal simulator of the areas of the city at higher risk of oxidative stress based on the association between greenness and 15-F2t-IsoP and overlapped to the actual number of children living in Turin. The dashed boxes indicate the areas that should be considered for a requalification.*

5.4. Bisphenol A exposure, age and oxidative stress in adolescents (Study line II)

Table 12 summarises the descriptive analyses of the sample population that has been sub-grouped by schools, thus age classes (primary school: 7-10 years; lower secondary school: 11-14 years and upper secondary school: 15-19 years). Overall, 153 subjects were non-smokers (68.7%), while 52 were passive smokers (23.3%). Only 18 adolescents (aged 14-19 years) reported being active smokers (8%).

School		Primary	Lower Secondary	Upper Secondary	Overall
Age group (yrs)		(7-10)	(11-14)	(15-19)	(7-19)
Numerousness n (%)		87 (39.0)	34 (15.3)	102 (45-7)	223 (100)
Gender n (%)	Male	48 (55.2)	15 (44.1)	57 (55.8)	119 (53.3)
	Female	39 (44.8)	19 (55.9)	45 (44.2)	104 (46.7)
Smokers n (%)	Active	0 (0.0)	0 (0.0)	18 (17.6)	18 (8.0)
	Passive	26 (30.0)	5 (14.7)	21 (20.5)	52 (23.3)
Age (yrs)		8.87 (1.0)	11.7 (0.8)	16.6 (1.71)	12.8 (3.8)
Height (m)		1.39 (0.08)	1.54 (0.1)	1.71 (0.08)	1.56 (0.17)
Weight (kg)		35.6 (9.8)	45.0 (7.5)	64.5 (12.4)	50.2 (17.2)

Notes: Weight, height and age are expressed as mean (SD)

Table 12 *Demographics of the sample population (n =223). Study line II*

The Piecewise regression tested the association between bisphenol A (log-transformed GlcA–BPA) and oxidative stress (log-transformed 15-F2t-IsoP) in the sample population, accounting for the age classes and tobacco smoking exposure (log-transformed cotinine) (**Figure 9 and table 13**). First, there was a breakpoint at 1.79 (95% C.I. from 1.56 to 2.02, $p < 0.001$) of the Log-transformed GlcA–BPA on log-transformed 15-F2t-IsoP, corresponding to 6 ng/mg Crea of GlcA-BPA. Second, the concentration of log-transformed 15-F2t-IsoP increased exponentially (more than threefold for each one-log unit of GlcA–BPA), when the log-GlcA–BPA concentration overcame the breakpoint. Third, a 12% increase of oxidative stress (log-transformed 15-F2t-IsoP) has been observed for each increase in one unit of the log-transformed cotinine.

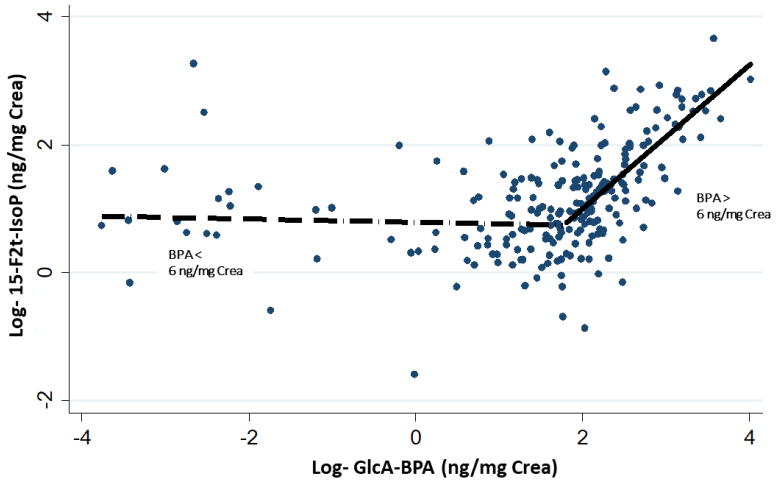


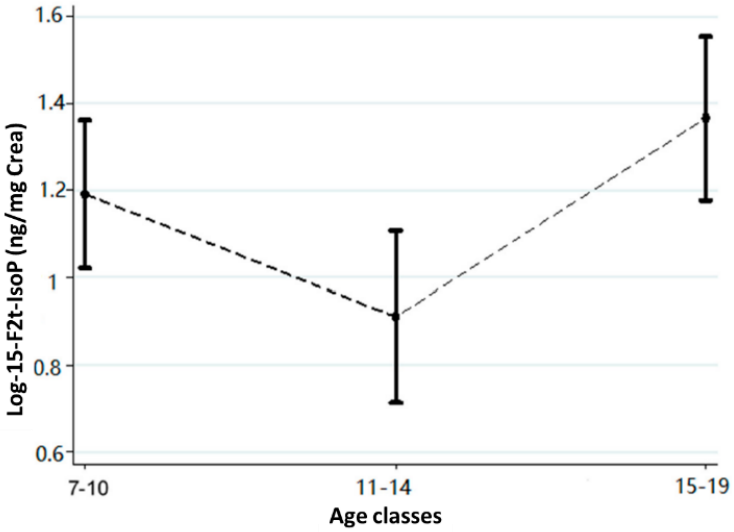
Figure 9 *The scatterplot reporting the association between oxidative stress (Log(15-F2t-IsoP)) and bisphenol A (Log (GlcA-BPA)) and the breakpoint at 1.79.*

Log (15-F2t IsoP)		Coeff. (95% C.I)	*P-value*
Breakpoint	Breakpoint	1.79 (1.56; 2.02)	**< 0.0001**
	Up to the breakpoint	-0.01 (-0.10; 0.08)	0.82
	Beyond the breakpoint	1.11 (0.87; 1.34)	**< 0.0001**
Age classes (yrs)	≤ 10	*Ref.*	
	11-14	-0.20 (-0.41; 0.00)	**0.05**
	≥ 15	-0.07 (-0.27;0.14)	0.53
Log (cotinine) (ng/mg Crea)		0.03 (0.00; 0.06)	**0.05**

Table 13 *The Piecewise regression used to test the association between bisphenol A (log-transformed GlcA–BPA) and oxidative stress (log-transformed 15-F2t-IsoP).*

Additionally, the association between age classes and oxidative stress highlighted that there was a significant decrease of the log-transformed 15-F2t-IsoP (p = 0.026) from the childhood stage (7–10 years) to the first adolescence phase (11–15 years) while, starting from 15 years, an increasing trend in oxidative stress was observed (**Figure 10**). Hence a V-shape distribution has been highlighted.

Figure 10 *The V-shape trend observed for the oxidative stress levels variations among the classes of age*



5.5. Non-invasive oxidative stress biomarkers in adults: systematic review and meta-analysis (Study line III)

Figure 11 depicts the step-by-step process used to carry out the systematic review. The number of records resulting from the search string launch has been reported (n = 4,479), detailing those that have been screened (n = 3,242), assessed for eligibility (n = 44) and included in the qualitative analysis (i.e. systematic review, n = 43) or in the quantitative analysis (i.e. meta-analysis, n = 21 divided into two meta-analyses). The majority of the studies (47%) was an uncontrolled experiment employing a before-after design (n = 20), while a cumulated 49% consisted of controlled before-after, longitudinal and randomised controlled trials (n = 7, respectively). One study used a randomised cross-over design and another was a self-controlled case-series. Studies were mainly implemented in Japan (n = 4), Brazil (n = 4), Italy (n = 4), Spain (n = 4), and USA (n = 4) followed by Canada (n = 3), Netherlands (n = 3), Iran (n = 2) and Denmark, Egypt, Germany, Greece, India and Mexico (n = 1, respectively). Nine studies did not clearly state the study location, but have been set up and drafted by authors whose affiliations were mixed such as Denmark and United Kingdom (n = 1), Greece and Cyprus (n = 3) Germany and Austria (n = 1), Hungary and Canada (n = 1), Iran and Japan (n = 1), USA and Italy (n = 1) and USA and Canada (n = 1). The studies were published during the time-lapse comprised between 1993 and 2019, 12% of which in 2014.

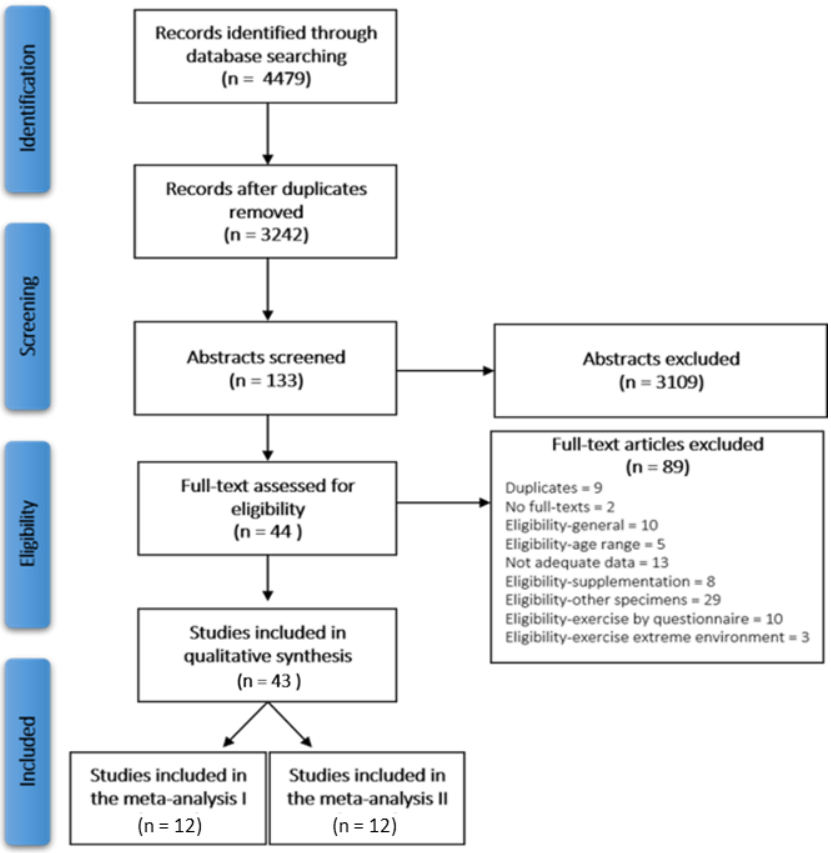


Figure 11 *PRISMA flowchart that summarises the whole systematic review process, step-by-step*

Among the 43 studies included in the qualitative analysis, the two most investigated oxidative stress biomarkers were isoprostanes and 8-oxo-dG or 8-OH-dG. In particular, 42% of the studies focused on 8-oxo-dG or 8-OH-dG (n = 18), 40% analysed the isoprostanes (n = 17), and the remaining 17% included a large variety of other biomarkers (i) measured in saliva, such as peroxidase, lipid hydroperoxide, superoxide dismutase, total antioxidant status or capacity, advanced oxidation protein products, glutathione, vitamin C and uric acid; (ii) measured in urine, such as allantoin, hydrogen peroxide, and urate; (iii) measured in both urine and saliva such as malondialdehyde, which has been analysed in 7 studies out of 43 (16%). The majority of the studies (84%) quantified the oxidative stress biomarkers in urine, either using spot urine (n = 20), either 24-h (n = 11) or 12-h urine (n = 4), while 6 articles out of 43 have used saliva specimens. The most widely performed analytical technique was ELISA (59% of the studies), followed by HPLC (23%), while the remaining 28% of the studies used radioimmunoassay (n = 2), GC-MS, tandem mass spectrometry and ultra-performance liquid chromatography (n = 1, respectively) or other analytical techniques generally defined as “colorimetric” (n = 2), “spectrophotometric” (n = 2), “enzymatic” (n = 1) or even not declared (n = 2).

In terms of the sample population, 957 adults aged between 19 and 72 years (39.8 ± 18.2 years) were included in the systematic review, and 71% of them were males (n = 675). The majority of the subjects was healthy (60%), 10% were not clearly classified, while 30% reported a disease among diabetes or obesity (13%), respiratory diseases (9%) and a miscellaneous of other pathologies (8%). The enrolled subjects were generally active (41%) or professional athletes (24%), whereas 33% of them reported a sedentary lifestyle and 2% did not provide any details. Studies also differed by the physical activity protocol applied, since only 27% of the subjects performed low-intensity physical activity and the remaining participants were engaged in high-intensity (42%) or medium-intensity (25%) physical activity protocols (for 6% of them the physical activity intensity has not been specified). Disregarding the intensity, also the types of physical activity were different including 5% of subjects who underwent both aerobic and anaerobic exercises, while the remaining subjects followed an aerobic (67%) or anaerobic (27%) protocol.

The studies included in the qualitative analysis (n = 43) reported a large number of different oxidative stress biomarkers, thus a lack of homogeneity. Therefore, only the studies focusing on the two most frequently investigated biomarkers were selected and included in the meta-analysis. Hence, two different meta-analyses were carried out (1) the meta-analysis I, focusing on the effect of physical activity on 8-oxo-dG or 8-OH-dG; (2) the meta-analysis II, focusing on the effect of physical activity on isoprostanes. Indeed, investigating isoprostanes or 8-oxo-dG or 8-OH-dG may provide valuable information in terms of different oxidation pathways that can be implicated in exercise-induced oxidative stress, namely lipid peroxidation and DNA oxidation.

5.5.1. Meta-analysis I: physical activity and urinary 8-OH-dG and 8-oxo-dG

Figure 12 shows the Forest plot relying on the meta-analysis conducted including 12 eligible studies, which provided an overview on the summary effect, heterogeneity and the outliers. Data from those studies labelled by “a” and “b” letters were included accounting for each sub-group that had been published by the author, thus provided as clusters instead of pooled data. The studies that provided only sub-grouped data based on grouping criteria that were not relevant to the research question of the systematic review (e.g. smokers *vs* non-smokers), were excluded from the meta-analysis, since they might have added further heterogeneity.

The overall effect was not statistically significant and the total fixed and random effects did not agree (-0.19, 95% CI -0.34 to -0.04 and +0.03, 95% CI -0.92 to +0.98, respectively). Moreover, a substantial heterogeneity was detected (I^2 = 97%, $p < 0.001$). Therefore, a set of sensitivity analysis was performed. Firstly, the outliers have been inspected and removed, thus only a few studies were found to substantially contribute to the overall effect; secondly, the contribution of the single study to the overall heterogeneity has been explored, detecting that *Allgayer et al.*, *Poulsen et* al., and *Parise et al.* contributed the most; thirdly, the publication bias was evaluated both graphically, by using the Funnel plot (**figure 13**), and by Egger’s test (p = 0.83), founding no asymmetry, thus no publication bias. Finally, several meta-regressions were carried out accounting for the potential effect of other predictors (e.g. participant’s characteristics, sub-groups, physical activity type, duration and intensity). However, none of these analyses was able to explain the substantial heterogeneity.

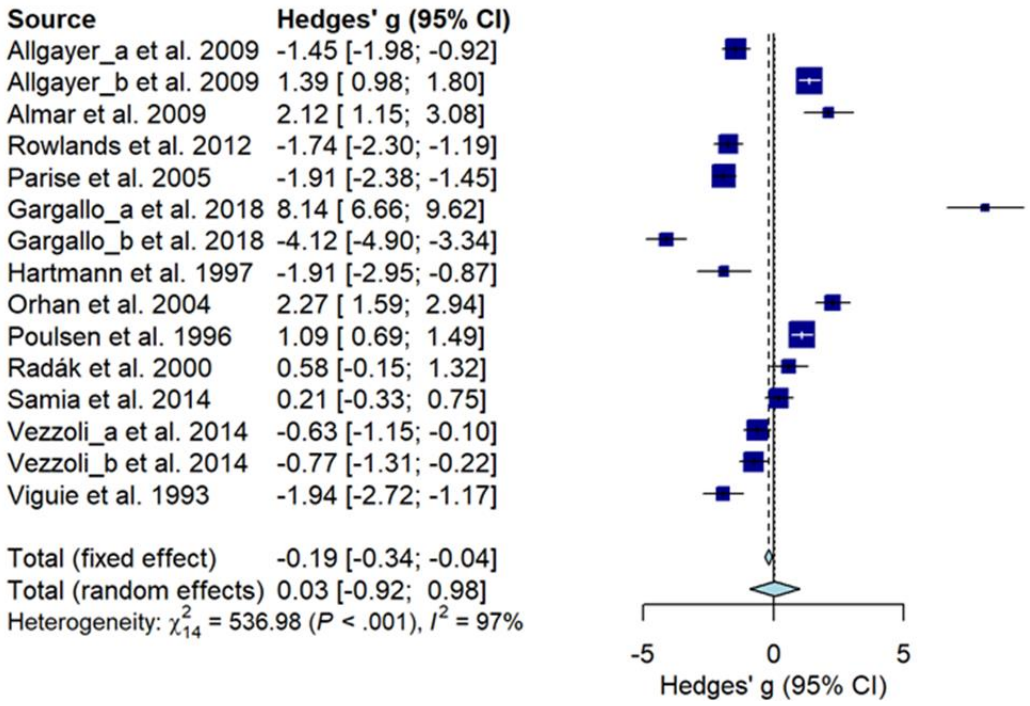


Figure 12 *The Forest plot that summarises the results of meta-analysis I on 8-oxo-dG and 8-OH-dG biomarkers*

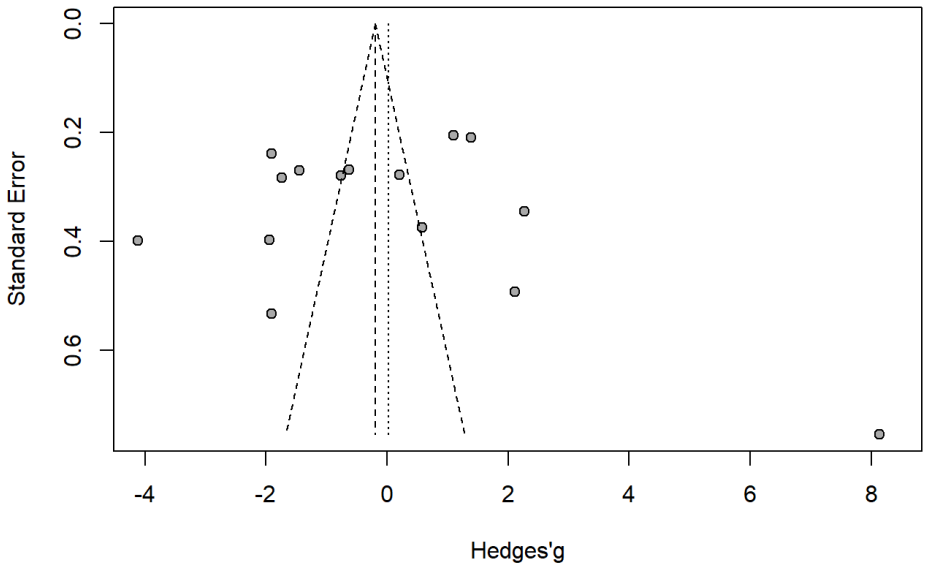


Figure 13 *The Funnel plot that shows the absence of publication bias in the meta-analysis I*

5.5.2. Meta-analysis II: physical activity and urinary isoprostanes

The meta-analysis on isoprostanes followed a similar methodology already presented for that on the DNA oxidation biomarkers in the previous paragraph. **Figure 14** shows the Forest plot referred to the meta-analysis conducted including 12 eligible studies.

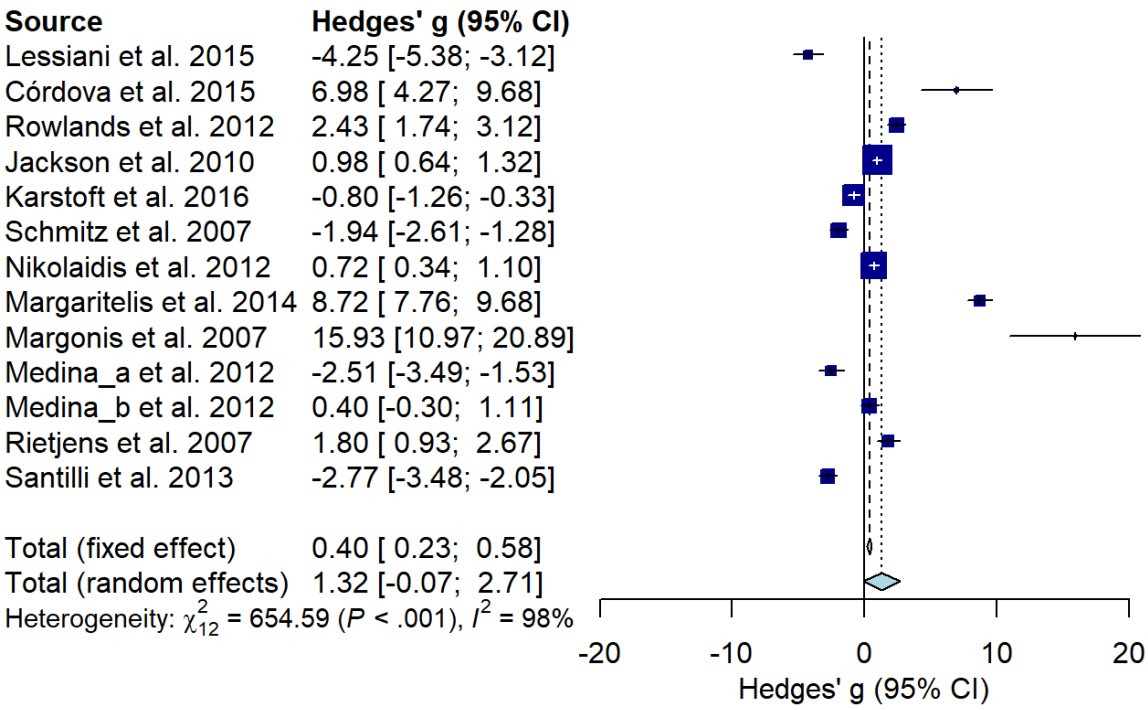


Figure 14 *The Forest plot that summarises the results of meta-analysis II on isoprostane biomarkers*

The combined effect of the meta-analysis underlined that the isoprostane induction is stimulated by physical activity (Total fixed effects: +0.40, 95% CI 0.23 to 0.58; total random effects: +1.32, 95% CI -0.07 to 2.71), but it is barely significant. Due to the substantial heterogeneity (I^2 = 98%, p < 0.001), cautiousness should be paid when interpreting this summary result. Based on the aforementioned heterogeneity, further analysis have been performed to (i) inspect the outliers; (ii) explore the contribution of the single study to the overall heterogeneity, detecting that *Margaritelis et al.* contributed the most ; (iii) evaluating the publication bias both graphically, by using the Funnel plot (**figure 15**), and by Egger's test (p = 0.60), founding no asymmetry, thus no publication bias; (iv) further, several meta-regressions were carried out accounting for the potential effect of other predictors (e.g. participant's characteristics, sub-groups, physical activity type, duration and intensity). However, none of these analyses was able to explain the substantial heterogeneity.

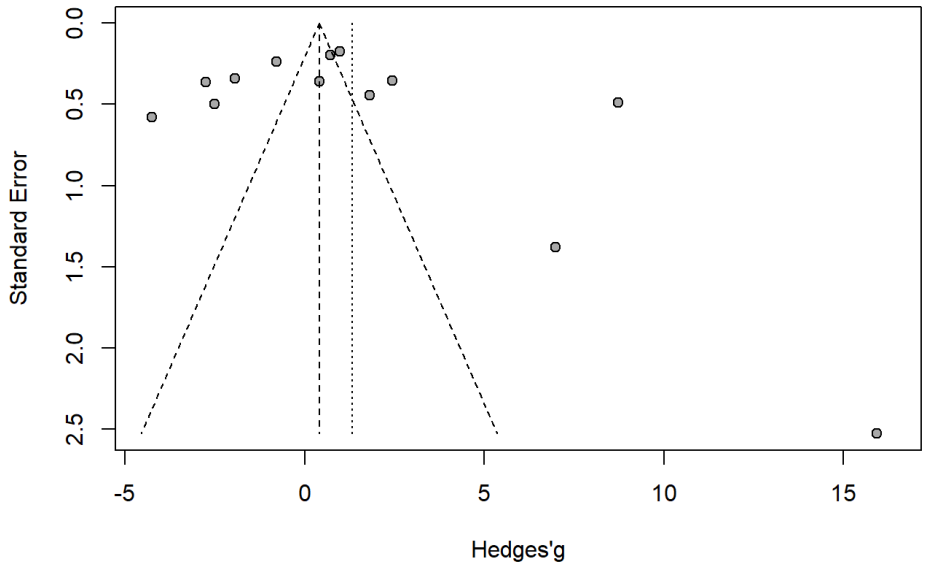


Figure 15 *The Funnel plot that shows the absence of publication bias in the meta-analysis II*

6. GENERAL DISCUSSION

The general purpose of this doctoral thesis was to investigate whether different domains, namely individual, environmental and behavioural domain, could affect oxidative stress in children, adolescents and adults. Therefore, several acknowledged or supposed oxidative stress inductors have been selected from each of the aforementioned domain (i) BMI-based obesity and age; (ii) exposure to greenness and to bisphenol A and (iii) tobacco smoking and physical activity. All these factors have been analysed in subjects with different age, who were living in differently-urbanised areas of the Po Valley, in northern Italy. A molecular epidemiology approach was used to investigate the association between the selected factors and oxidative stress. This latter was measured by the urinary 15-F2t-IsoP, which is one of the most commonly used oxidative stress biomarker in human studies, able to reflect the systemic oxidative *status* derived from the *in vivo* lipid peroxidation reactions.

6.1. Main results

The main findings of this PhD thesis are summarised and discussed afterwards according to the previously presented study lines and domains.

6.1.1. Study line I

From the individual domain standpoint, body composition and, in particular, **obesity** condition assessed by BMI, influences oxidative *status* in healthy children by increasing urinary 15-F2t-IsoP. Being obese promotes an increase of 56% in 15-F2t-IsoP levels compared to those measured in normal-weight children (p = 0.020). Additionally, the BMI-based characterisation of body composition deserves carefulness. The prevalence of youths classified as overweight or obese varies considerably depending on the selected BMI cut-off points or centiles that is specific to each BMI reference standard. The three selected BMI standards highlighted significant differences in the prevalence of underweight, normal weight, overweight and obese. Indeed, the application of the IOTF standard produced the smallest percentage of obese children compared to the CDC standard (14% vs 21%) and even a wider difference compared to the WHO standard (14% vs 34%) was observed. These results are in keeping with those reported by other studies (Gonzalez-Casanova et al. 2013; Kêkê et al. 2015; Valerio et al. 2017) . The IOTF standard was more accurate in categorising obese children and more appropriate in oxidative stress investigations. Indeed, the statistical analyses that took into account the other two BMI reference standards did not show any statistically significant associations between obesity and oxidative stress. Only the IOTF standard seems to clarify the role of adiposity in oxidative stress induction, showing an appropriate accuracy, independently from age, sex, gender, physical activity frequency and tobacco smoking exposure.

These findings are generally in agreement with the previously published literature. In particular, previous research has shown that there is a lack of consistency among different international BMI reference standards in assessing overweight and obesity in children and adolescents (Gonzalez-Casanova et al. 2013; Shields and Tremblay 2010). In 2015, Medehouenou and colleagues reported that the IOTF standard was more accurate in categorising 290 Inuit school-aged children as overweight or obese (Medehouenou et al. 2015) and the same was observed in 1382 children enrolled in France (Kêkê et al. 2015). In general, significant positive correlation has been observed between higher BMI and oxidative stress biomarkers in children (Codoñer-Franch et al. 2011; Fernández-Sánchez et al. 2011; Marseglia et al. 2014)

Various mechanisms can explain the obesity-related oxidative stress induction. First, several interrelated pathophysiologic pathways, at cellular and metabolic levels, including the metabolic overload, the mitochondrial dysfunction, and endoplasmic reticulum stress. The metabolic overload occurs in overweight and obese subjects whose caloric intake exceeds the energy expenditure

inducing an increase in Krebs's cycle activity, thus an increase in ROS production (Codoñer-Franch et al. 2011); hyperglycaemia could be considered in the group of metabolic alterations being able to activate a large number of oxidative pathways. The mitochondrial overproduction of ROS occurs into adipocyte mitochondria, where the excess of free fatty acids causes the mitochondrial uncoupling and the consequent increase of ROS (Gao et al. 2010). The endoplasmic reticulum stress happens when a higher demand in protein folding, which should be supported by the endoplasmic reticulum, is required (Codoñer-Franch et al. 2011). Second, the adipose tissue has been recognised as an active endocrine organ directly involved in the production of adipocytokines or adipokines, interconnecting oxidative stress with inflammation (Hensley et al. 2000). Third, increased muscle activity, as one of the consequences of carrying an excessive weight, could also increase the electron transport chain activity and the respiration rate increasing, in turn, the ROS production (Saiki et al. 2001). Fourth, an inadequate antioxidant response has been observed in obese subjects, who usually reduce the anti-oxidant intake, normally deriving from a balance diet, but also have lower endogenous anti-oxidant concentrations (i.e. beta-carotene, alpha-tocopherol, vitamin C) compared to non-obese (Strauss 1999; Vincent et al. 2010). Finally, obesity determines the endothelial dysfunction (Manna and Jain 2015) and a progressive infiltration of macrophages into the adipose tissue, which promote the increase of oxidative stress (Vincent and Taylor 2006).

Concerning **passive tobacco smoking** exposure, it was acknowledged as another important predictor of oxidative stress induction in healthy children. In particular, children belonging to the third and the fourth cotinine quartiles showed higher 15-F2t-IsoP levels (+1.45, 95% CI 1.06 to 1.97 ng/mg Crea, $p = 0.020$ and +2.04, 95% CI 1.55 to 2.69 ng/mg Crea, $p < 0.0001$, respectively) compared to those laying in the first quartile, highlighting that even low-grade exposures to passive tobacco smoking may induce an oxidative stress increase in healthy children.

This finding is consistent with previous scientific evidence reporting that the exposure to passive tobacco smoking induces oxidative stress (Al-Sayed and Ibrahim 2014; Bono et al. 2015; Flouris et al. 2010; Goel et al. 2018; Groner et al. 2016; Valavanidis et al. 2009b). Aycicek (Aycicek et al. 2005) reported higher antioxidant levels among young infants aged between 6–28 weeks who were exposed to five cigarette per day. Kosecik and colleagues pointed out that plasmatic oxidative stress biomarkers were increased in exposed children compared to unexposed subjects (Kosecik et al. 2005). Exposure to cigarette smoking can act through direct and/or indirect mechanisms (i) by exposing subjects to pro-oxidant chemicals contained into the cigarette smoke (Flouris et al. 2010)and (ii) by impairing the endogenous antioxidant defence systems (Flouris et al. 2010; Valavanidis et al. 2009b). Indeed, the large number of free radicals contained in cigarette smoke can be produced during the burning of tobacco or are generated from the initial total particulate matter dissolved in the biological media.

With regard to the environmental domain, **greenness** exposure was associated to oxidative stress in children. In particular, higher **NDVI** values relate consistently to lower oxidative stress levels, although most associations were not statistically significant. All greenness variables were inversely associated with oxidative stress (log-transformed 15-F2t-IsoP), attesting a decrease in oxidative stress for each 0.01 unit of increase in average NDVI (β: -0.39, 95% CI -0.85 to 0.05), in vegetated portion (β: -0.28, 95%CI -0.61 to 0.04), in time-weighted average NDVI (β: -0.63, 95%CI -1.27 to 0.02) and in time-weighted vegetated portion (β: -0.50, 95%CI -0.98 to -0.02). Additionally, children reporting the lowest and highest frequencies of physical activity were those who had also the higher levels of oxidative stress (β: +0.19, 95%CI 0.04 to 0.43; β: +0.25, 95%CI 0.04 to 0.46) compared to the moderately-active children. A second finding concerns **physical activity frequency** that could partially explain the observed association between NDVI and oxidative stress, as including it in the model attenuated the association for greenness variables.

The main findings are in agreement with previous reports, which however did not analysed children population. In a cross-sectional study, NDVI surrounding participants residence in Kentucky (n = 82, adults) was inversely associated with oxidative stress, independently from age, sex, race, smoking status, neighbourhood deprivation, and roadway exposure (Yeager et al. 2018). Longer telomeres, whose length represents a sort of cellular memories of oxidative stress and inflammation (Martens and Nawrot 2018), have been found in a cohort of 976 elderly living in greener areas of Hong Kong (Woo et al. 2009) and in Belgian new-born twins (n = 211) whose telomere length was prospectively and positively associated with maternal residential greenness (Bijnens et al. 2015). On the contrary, the current thesis is based on a children sample population, that has not previously investigated, and on a multisite exposure, which improved the exposure characterisation. Previous literature highlighted that greenness and physical activity are associated, both in youths and adults (James et al. 2015). In a cohort study of 365 pre-schoolers from Illinois, increased physical activity was associated with higher NDVI (Grigsby-Toussaint et al. 2011). Similar results were found in 3042 adolescents from Wesel (Markevych et al. 2016) and in 69,910 young Canadian adults (McMorris et al. 2015). In the current PhD thesis both more active children (≥ 4 days/week at least 60 min.) and those engaged in lower physical activity frequencies (0-1 days/week at least 60 min.) showed the highest levels of oxidative stress compared to children who exercised up to 2 days per week. This is in agreement with previous data reporting that higher physical activity frequency is linked to chronic oxidative insults, while low frequency lacks in stimulating the anti-oxidant defence systems, and only mild frequency is able to stimulate the repair pathways counteracting oxidative stress in young adults (Pittaluga et al. 2006).

The biological mechanisms underlying the effect of greenness exposure on oxidative stress could include (i) the immune-system regulation that greenness may exert promoting the exposure to some categories of organisms that normally colonise natural environments (Rook 2013), known for stimulating the immune system development in children (Aerts et al. 2018); (ii) higher exposure to greenness that occurs outdoors may increase the exposure to sunlight, whose ultraviolet B rays' component directly stimulates the synthesis of Vitamin D, which is a known anti-oxidant (Wimalawansa 2019) also in youth (Filgueiras et al. 2020), (iii) the increased exposure to greenness can promote outdoor physical activity, which in turn affects oxidative stress (Powers et al. 2020) and (iv) higher greenness implies a great many trees surrounding the living environment that are able to act as a buffer against the airborne toxicants, thus to counteract the air pollution pro-oxidant effects.

6.1.2. Study line II

The association between **greenness** and oxidative stress that was detected in children has been also observed in adolescents. In this case, higher residential exposure to **SAVI** inversely relates to oxidative stress. Additionally, where the evergreen trees dominate oxidative stress is lower. Indeed, a significant negative correlation was found between 15-F2t-IsoP-Ev and PGC-Ev ($r = -0.758$, $p < 0.001$) and between 15-F2t-IsoP-Ev and uSAVI-Ev ($r = -0.717$, $p < 0.001$) indicating that higher vegetation cover seems to significantly reduce oxidative stress in adolescents. The same result was confirmed by the corresponding bivariate regression models. Moreover, NET and NBT bivariate models gain values were significantly different with NET determining a negative steeper 15-F2t-IsoP-Ev reduction rate (six-time greater compared to NBT), suggesting that evergreen trees determine a stronger positive effect on oxidative stress.

No previous results have been published on this topic, thus any comparisons with the existing literature is not feasible.

The mechanisms underlying the association between greenness and oxidative stress have been already mentioned in the previous paragraph. However, the effect of **evergreen trees** on the redox

status needs additional clarification. Trees play an important role in improving air quality by facilitating the dry deposition of the gaseous pollutants, which are trapped into the leaf *stomata*, and also the particulate pollutants, mainly retained by the tree canopy surfaces (Hirabayashi and Nowak 2016). Nevertheless, the removal efficiency varies among tree species attesting a remarkable efficacy among the evergreen trees. For example, some conifers can be more effective than broadleaf trees based on their (i) year all around *foliage*; (ii) fast growth rate; (iii) dense canopy and (iv) resinous leaves (Yang et al. 2015).

Another important feature of the environmental domain was the ubiquitous **bisphenol A**, whose exposure strongly relates to oxidative stress induction in adolescents. Moreover, only the exposure higher than a threshold value (breakpoint of 1.79, corresponding to 6 ng/mg Crea of GlcA-BPA) seems to determine a linear increase in 15-F2t-IsoP ($p < 0.005$). The age of the enrolled subjects has been identified as another influencing factor that significantly affects oxidative stress, which slightly decreases in the 11-14 age class compared to the others, namely 7-10 and 15-19 years.

The observed association between exposure to bisphenol A and oxidative stress is in keeping with previous studies. In a recent systematic review, it has been reported that the most frequently measured biomarkers of oxidative stress were 8-OH-dG, 15-F2t-IsoP and malondialdehyde, which almost always were positively associated with bisphenol A exposure (Steffensen et al. 2020). In a cohort study by Zhou and colleagues (Zhou et al. 2019) including 275 Chinese school-aged children, increased oxidative stress was significantly associated with bisphenol A exposure. The same result was observed in a cross-sectional study involving 300 Brazilian children (6-14 years old) (Rocha et al. 2018)Another cross-sectional study reported that the exposure to bisphenol A determined a significant increase in oxidative stress in 96 children aged between 3 and 6 years (Lv et al. 2016).

The mechanisms underlying the bisphenol A toxicity may be related to its chemical properties and its metabolization for the consequent detoxification once in the body. First, bisphenol A circulating in the body stimulates the ROS production by the enzymatic and non-enzymatic formation of phenoxyl radicals (Gassman 2017). Subsequent reactions of these radicals along with further enzymatic processing produce a variety of additional radical species, including superoxide, peroxide, and hydroxyl radicals (Babu et al. 2013). Second, it can determine the depletion of the anti-oxidant defence systems, because the quantity of ROS generated by the metabolization may exceed the capacity of the intracellular anti-oxidants. Third, bisphenol A can induce the inhibition of the mitochondrial energy production (Nakagawa and Tayama 2000), by depleting intracellular ATP. Finally, it can alter some cell signalling pathways contributing to the generation of ROS (Rochester 2013). In particular, the activation of the mitogen activated protein kinase (MAPKs), PI3K/AKT, and NF-jB pathways have been implicated in BPA-induction of oxidative stress and inflammation (Zhu et al. 2015).

6.1.3. Study line III

The **meta-analysis I** on 8-OH-dG and 8-oxo-dG, did not highlighted any pooled effect of physical activity on these biomarkers; thus, the contradictory among the included articles was not solved. The total fixed and random effects were not in agreement and did not reach the significance level (-0.19, 95% CI -0.34 to -0.04 and +0.03, 95% CI -0.92 to +0.98, respectively). Moreover, a substantial heterogeneity was detected (I^2 = 97%, $p < 0.001$). Despite the risks of a misleading interpretation when analysing the overall effect, some study-level remarks could be made. Indeed, some subgroups highlighted that there was a different DNA oxidation response depending on the physical activity intensity. In *Allgayer et al*., DNA oxidation was decreased in active patients with cancer who performed moderate physical activity (group a) compared to active patients performing high-intensity physical activity (group b). The same trend was observed in *Gargallo et al.* where moderate exercise reduced the oxidative stress levels in healthy sedentary subjects (group b) compared to

those engaged in intense physical activity protocol (group a). These findings indicate that a moderate physical activity intensity could support the maintenance of the health *status*, avoiding the oxidative stress overproduction. On the other hand, the **meta-analysis II,** that focused on isoprostanes, highlighted that urinary isoprostanes are more prone to be influenced by an acute bout of physical activity, although this result barely reached the significance level but it is barely significant (Total fixed effects: +0.40, 95% CI 0.23 to 0.58; total random effects: +1.32, 95% CI -0.07 to 2.71) and a substantial heterogeneity has been detected (I^2 = 98%, $p < 0.001$).

Some physiological mechanisms can explain how physical activity can affect oxidative stress and, in particular, why moderate exercise is advisable. In general, a bout of physical exercise impairs the redox homeostasis by inducing an increase in ROS production. However, an optimum level of ROS induced by physical activity may exert a beneficial function initiating a series of adaptive changes in the stressed system (Alessio and Blasi 1997; Powers et al. 2016). They can modulate transcription factors and gene expression. For example, a transient increase of the exercise-induced oxidative stress can initiate redox-sensitive signalling pathways (e.g. PPARγ, PGC1- α, MAPK, etc.) that contribute to both training adaptations and general health benefits (Fisher-Wellman and Bloomer 2009; Radak et al. 2005; Webb et al. 2017). Further, exercise-induced ROS are able to increase the antioxidant enzymes activity (e.g. superoxide dismutase, glutathione peroxidase, etc.), leading to an enhancement of the endogenous antioxidants (Boccatonda et al. 2016). Indeed, habitual and moderate exercise serves as a necessary *stimulus* in the up-regulation of the anti-oxidant defences and beneficial adaptations, while strenuous and sporadic exercise promote harmful oxidative stress (Powers et al. 2020).

6.2. Strengths and limitations

The current PhD thesis has several strengths. First and foremost, oxidative stress was quantified by means of 15-F2t-IsoP, which is a reliable and acknowledged biomarker of oxidative stress quantification *in vivo* (Musiek et al. 2005).

From the individual domain standpoint, a comparison between three international and already validated BMI reference standards was made, indirectly testing their accuracy in categorising children as obese with the final purpose of investigating oxidative stress. Concerning the environmental domain, the main point was the novelty in assessing the influence of greenness on oxidative stress in children. Very few studies analysed this association, however they did not focus on children, nor have used other metrics than NDVI or a multi-site exposure (i.e. time-weighted greenness variables). This latter accounted for the partial movement throughout the day, reducing exposure misclassification. With regard to vegetation metric and quantification, is important to notice that both NDVI and SAVI were calculated at individual level using a 10 x 10 m image resolution and images were calibrated accounting for earth reflectance, which considerably improve their calculations. Additionally, different buffers have been used to characterise exposure to greenness to avoid leaving out the optimal distance of impact on oxidative stress, which has not been established yet in children. Further, we included children from different schools and found no evidence of heterogeneity, indirectly supporting absence of residual confounding. This doctoral thesis mainly focused on children and adolescents, whose susceptibility deserves particular attention, especially in preventive terms, and gave an insight into some environmental key features able to interfere with their healthy development (i.e. bisphenol A exposure). Concerning the study line III, it is important to remark that the systematic review was carried out following a rigorous procedure (i) avoiding additional bias during the entire process, since an *a priori* protocol was drafted and published; (ii) conducting a double-blind selection of the studies, by two independent reviewers; and (iii) assessing the risk of bias of the included articles, using validated quality assessment tools based on the study designs of the included studies.

The main limitation of the studies included in this thesis lays into their cross-sectional design that did not permit to conclude on causal relationship between independent variables and oxidative stress. Potential selection bias may deserve consideration, as the participants were enrolled as volunteers. However, it is worth mentioning that all education levels were represented and all participants were healthy, as inclusion criteria. A set of covariates were considered and included in the analyses nonetheless residual confounding is still possible due to potentially unmeasured confounders known to be related to oxidative stress. Physical activity and parental education, were assessed by questionnaire, which may lead to misclassification or recall bias, although a standardised questionnaire has been used. Finally, this study may have limited external validity since children were enrolled from a single geographic area (northern Italy).

6.3. Implications for research

The current PhD thesis provides several original results ranging from the application of already validated tools to the exploration of new instruments and preventive grids in supporting Public Health. First, BMI remains useful due to its easy measurement, cost-effectiveness, and high correlation with other tools for body composition assessment in epidemiologic studies (Lønnebotn et al. 2018). However, IOTF reference standard may be more helpful in supporting further research on oxidative stress investigations and obesity in children. Whereas all three BMI standards are effective tools in prevention strategies, their differences in accuracy, sensitivity, and specificity requires specific fields of application. Second, the impact of greenness on certain health outcomes has been linked to biogeographic characteristics (Fuertes et al. 2016; Tischer et al. 2017) and based on the paucity of studies on the Italian population, this work may be a valuable contribution providing empirical data on this topic, which is still under-investigated. Future research is needed to define the mechanisms behind this association, possibly considering a longitudinal design, which allows to infer on the long-term effects of greenness exposure and on the mediating role of physical activity. Third, further research is needed to elucidate the safeness of plastic materials whose content in bisphenol A is already under restriction. Possibly, leaching analysis from plastic materials and the consequent effect on oxidative stress should be taken into consideration for future implementations. Finally, future research on exercise-induced oxidative stress biomarkers should rather prefer a set of biomarkers instead of just one, since they may display different behaviours.

6.4. Implications for stakeholders

These findings would be of interest for Public Health interventions and policy decision makers. First, they can support better prevention strategies against obesity and risky conditions in children, highlighting that BMI represents a suitable tool in epidemiologic investigations on oxidative stress. Second, they might help urban green spaces policies and reinforce the awareness on the contribution that physical activity and lifestyles play in health and well-being, especially in susceptible populations as children. Finally, they could stimulate the consciousness of the need of safer materials which may produce least impact on the environment and human health.

CONCLUSIONS

In conclusion this doctoral thesis highlighted that (i) BMI-based obesity is associated with increased oxidative stress in children and the IOTF seems to be more accurate in categorising children as obese compared to the other selected BMI reference standards; (ii) even a low-grade exposure to passive tobacco smoking is able to induce oxidative stress increase in healthy children; (iii) greenness exposure is associated with decreased oxidative stress in children and physical activity could partly mediate this relationship; (iv) the exposure to bisphenol A can induce oxidative stress from a specific exposure level (6 ng/mg Crea of GlcA-BPA); (v) the quantification of a set of oxidative stress biomarkers instead of just one is advisable when investigating exercise-induce oxidative stress, even if isoprostanes seems to be more prone to increase after an acute bout of physical activity; and (vi) moderate physical activity seems to be more effective in reducing oxidative stress levels in adults compared to strenuous exercise.

Altogether these findings may represent valuable support for future research in the field providing novel evidence basis for better preventive practices in primary prevention and Public Health.

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ANNEX

Annex I - List of scientific contributions

List of manuscripts co-authored by the PhD candidate on oxidative stress induction in humans – *already published*

1. **Squillacioti, G**.; Bellisario, V.; Grosso, A.; Ghelli, F.; Piccioni, P.; Grignani, E.; Corsico, A.; Bono, R. Formaldehyde, Oxidative Stress, and FeNO in Traffic Police Officers Working in Two Cities of Northern Italy. Int. J. Environ. Res. Public Health 2020, 17, 1655.
2. Bono, R.; Bellisario, V.; Tassinari, R.; **Squillacioti, G**.; Manetta, T.; Bugiani, M.; Migliore, E.; Piccioni, P. Bisphenol A, Tobacco Smoke, and Age as Predictors of Oxidative Stress in Children and Adolescents. Int. J. Environ. Res. Public Health 2019, 16, 2025.
3. **Squillacioti, G**.; Bellisario, V.; Grignani, E.; Mengozzi, G.; Bardaglio, G.; Dalmasso, P.; Bono, R. The Asti Study: The Induction of Oxidative Stress in A Population of Children According to Their Body Composition and Passive Tobacco Smoking Exposure. Int. J. Environ. Res. Public Health 2019, 16, 490.

List of manuscripts co-authored by the PhD candidate on oxidative stress induction in humans – *under-review*

1. Geomatics and Epidemiology: Associating Oxidative Stress and Greenness in Urban Areas – De Petris, S.*; **Squillacioti, G**.*; Bono, R.; Borgogno-Mondino, E. (Environmental research)
2. Residential and school greenness exposure and oxidative stress in children. A cross-sectional study – **Squillacioti, G**.; Carsin, A.E.; Bono, R.; Garcya-Aymerich, J. (Environmental health perspectives)
3. Oxidative stress induction in woodworkers occupationally exposed to wood dust and formaldehyde – Ghelli, F.; Bellisario V.; **Squillacioti, G**.; Grignani, E.; Garzaro, G.; Buglisi, M.; Bergamaschi, E.; Bono, R. (Journal of Occupational Medicine and Toxicology)
4. Non-invasive biomarkers to quantify exercise-induced oxidative stress in saliva and urine. A systematic review - **Squillacioti, G**.; Colombi, N.; Guglieri, F.; Ghelli, F.; Berchialla, P.; Gardois, P; Bono; R. (Under preparation)

List of manuscripts co-authored by the PhD candidate on related topics - *already published*

1. Bellisario, V.; Piccioni, P.; Bugiani, M.; **Squillacioti, G**.; Levra, S.; Gulotta, C.; Mengozzi, G.; Perboni, A.; Grignani, E.; Bono, R. Tobacco Smoke Exposure, Urban and Environmental Factors as Respiratory Disease Predictors in Italian Adolescents. Int. J. Environ. Res. Public Health 2019, 16, 4048.
2. Bédard, A.; Carsin, A.-E.; Fuertes, E.; Accordini, S.; Dharmage, S.C.; Garcia-Larsen, V.; Heinrich, J.; Janson, C.; Johannessen, A.; Leynaert, B.; Sánchez-Ramos, J.S.; Peralta, G.; Pin, I.; **Squillacioti, G.**; et al. Physical activity and lung function—Cause or consequence? PLoS One 2020, 15, e0237769.
3. Carsin, A.-E.; Keidel, D.; Fuertes, E.; Imboden, M.; Weyler, J.; Nowak, D.; Heinrich, J.; Erquicia, S.P.; Martinez-Moratalla, J.; Huerta, I., Sanchez, J.L.; Schaffner, E.; Caviezel, S.; Beckmeyer-Borowko, A.; Raherison, C.; Pin, I.; Demoly, P.; Leynaert, B.; Cerveri, I.; **Squillacioti G**.; et al. Regular Physical Activity Levels and Incidence of Restrictive Spirometry Pattern: A Longitudinal Analysis of 2 Population-Based Cohorts. Am. J. Epidemiol. 2020.
4. Peralta, G.P.; Marcon, A.; Carsin, A.-E.; Abramson, M.J.; Accordini, S.; Amaral, A.F.; Antó, J.M.; Bowatte, G.; Burney, P.; Corsico, A.; Demoly, P.; Dharmage, S.; Forsberg, B.; Fuertes, E.; Garcia-Larsen, V.; Gíslason, T.; Gullón, J.A.; Heinrich, J.; Holm, M.; Jarvis, D.; Janson, C.; Jogi, R.; Johannessen, A.; Leynaert, B.; Martínez-Moratalla Rovira, J.; Nowak, D.; Probst-Hensch, N.; Raherison, C.; Sánchez-Ramos, J.L.; Sigsgaard,T.; Siroux, V.; **Squillacioti, G**.; et al. Body mass

index and weight change are associated with adult lung function trajectories: the prospective ECRHS study. Thorax 2020, 75, 313–320.

5. Parmes, E.; Pesce, G.; Sabel, C.E.; Baldacci, S.; Bono, R.; Brescianini, S.; D'Ippolito, C.; Hanke, W.; Horvat, M.; Liedes, H.; Maio, S.; Marchetti, P.; Marcon, A.; Medda, E.; Molinier, M.; Panunzi, S.; Pärkkä, J.; Polańska, K.; Prud'homme, J.; Ricci, P.; Snoj Tratnik, J.; **Squillacioti, G.**; et al. Influence of residential land cover on childhood allergic and respiratory symptoms and diseases: Evidence from 9 European cohorts. Environ. Res. 2019, 108953.
6. Ferrari, M.; Piccinno, E.; Marcon, A.; Marchetti, P.; Cazzoletti, L.; Pirina, P.; Battaglia, S.; Grosso, A.; **Squillacioti, G.**; Antonicelli, L.; et al. Chronic bronchitis without airflow obstruction, asthma and rhinitis are differently associated with cardiovascular risk factors and diseases. PLoS One 2019, 14, e0224999.
7. **Squillacioti, G.**; Bellisario, V.; Levra, S.; Piccioni, P.; Bono, R. Greenness Availability and Respiratory Health in a Population of Urbanised Children in North-Western Italy. Int. J. Environ. Res. Public Health 2019, 17, 108.
8. Lytras T, Beckmeyer-Borowko A, Kogevinas M, Kromhout H, Carsin AE, Antó JM, Bentouhami H, Weyler J, Heinrich J, Nowak D, Urrutia I, Martínez-Moratalla J, Gullón JA, Pereira Vega A, Raherison Semjen C, Pin I, Demoly P, Leynaert B, Villani S, Gislason T, Svanes Ø, Holm M, Forsberg B, Norbäck D, Mehta AJ, Keidel D, Vernez D, Benke G, Jõgi R, Torén K, Sigsgaard T, Schlünssen V, Olivieri M, Blanc PD, Watkins J, Bono R, **Squillacioti G**, et al. Cumulative Occupational Exposures and Lung Function Decline in Two Large General Population Cohorts. Ann Am Thorac Soc. October 2020: AnnalsATS.202002-113OC. doi:10.1513/AnnalsATS.202002-113OC

List of manuscripts co-authored by the PhD candidate on related topics - *under-review*

1. Bisphenol A and S in the urine of new-borns: plastic for non-food use still without rules– Valeria Bellisario, V.; Cocchi, E.; Tassinari, R.; **Squillacioti, G**.; Musso, T.; Sottemano, S.; Zorzi, M.; Dalmasso, P.; Coscia, A.; Medana, C.; Bono, R. (Toxics)
2. Bronchodilator response and lung function decline - associations with exhaled nitric oxide with regard to sex and smoking status – Nerpin, E; Ferreira, D.S.; Weyler, J.; Schlunnsen, V; Jogi, R.; Semjen, C.R.; Gislasson, T.; Demoly, P.; Heinrich, J.; Nowak, D.; Corsico, A.; Accordini, S.; Marcon, A.; **Squillacioti, G.**; et al. (Clinical Experimental Allergy)
3. Genomic damage in human as consequence of the exposure to formaldehyde in the pathology ward. The role of some gene polymorphisms as possible modulators – Ghelli, F.; Bono, R.; Cocchi, E.; Buglisi, M.; **Squillacioti, G.**; Bellisario, V.; Santovito, A (Toxicology)

Congress contributions

1. "Greenness and physical activity as possible oxidative stress modulators in children" Squillacioti G., Carsin A.E., Borgogno-Mondino E., Bono R., Garcia Aymerich J., (**PRESENTING AUTHOR IN ORAL PRESENTATION**). *The 16th World Congress on Public Health, Rome 2020*
2. "Urbanization and greenness in HBSC survey: association with overweight and obesity in adolescents" Bellisario V., Bono R., **Squillacioti G.**, Caputo M., Gintoli I., Borracino A., Lemma P., Dalmasso P. The *16th World Congress on Public Health, Rome 2020 and to the 32nd Annual Conference of the International Society for Environmental Epidemiology (ISEE), Washington 2020*
3. "Urbanization and greenness in HBSC survey: association with life satisfaction and health complaints" Gintoli I., Bellisario V., **Squillacioti G.,** Caputo M., Borraccino A., Dalmasso P., Bono R., Lemma P. The *16th World Congress on Public Health, Rome 2020 and to the 32nd Annual Conference of the International Society for Environmental Epidemiology (ISEE), Washington 2020*

4. “Greater Risk of Asthma and Allergic Rhinitis, But Not Eczema, Associated with Living Close to Green Space in European Children. The Heals Project”. Annesi-Maesano I, Maesano CN, Baldacci S, Bono R, Brescianini S, D'Ippolito C, Hanke W, Horvat M, Liedes H, Maio H, Marchetti P, Marcon A, Medda E, Molinier M, Panunzi S, Pärkkä J, Polanska K, Prud'homme J, Ricci P, Sabel CE, Snoj Tratnik J, **Squillacioti G** et al. In: C25. EARLY LIFE EXPOSURES AS DETERMINANTS OF RESPIRATORY DISEASE. American Thoracic Society; 2020: A4613-A4613. MeetingAbstracts.A4613, doi:10.1164/ajrccm-conference.2020.201.1_. *The International Conference of the American Thoracic Society “(ATS), Philadelphia 2020*
5. “Urban vegetation, oxidative stress and respiratory health in a population of Italian healthy children”. **Squillacioti G.**, Borgogno Mondino E., Garcia-Aymerich J., Bellisario V., Bono R. (**PRESENTING AUTHOR IN ORAL PRESENTATION – AWARDED**). *Atti Le giornate della ricerca scientifica e delle esperienze professionali dei giovani: Società Italiana di Igiene, Medicina Preventiva e Sanità Pubblica (SItI) Roma 20-21 dicembre 2019. J. Prev. Med. Hyg. 2019, 60, E1.*
6. “Greenness effect on oxidative stress and respiratory flows in children”. Environ Epidemiol. 2019; 3:35-36. Squillacioti G., Bellisario V., Ghelli F., Piccioni, P., Borgogno-Mondino E., Bono R. doi: 10.1097/01.EE9.0000606016. 27176.f4 (**PRESENTING AUTHOR IN THE POSTER DISCUSSION SECTION**). The *31st Annual Conference of the International Society for Environmental Epidemiology (ISEE), Utrecht 2019*
7. “Oxidative stress induction in woodworkers exposed to wood dust and formaldehyde” Ghelli F., **Squillacioti G.,** Bellisario V., Bono R. Environ Epidemiol. 2019;3:35. doi:10.1097/01.EE9.0000606012.89057.c0. The *31st Annual Conference of the International Society for Environmental Epidemiology (ISEE), Utrecht 2019*
8. “Oxidative Stress Profile of workers exposed to formaldehyde in the hospital” Bellisario V., **Squillacioti G.**, Ghelli F., Bono R. Environ Epidemiol. 2019;3:35. doi:10.1097/01.EE9.0000606008.81434.75. The *31st Annual Conference of the International Society for Environmental Epidemiology (ISEE), Utrecht 2019*
9. “Influence of the proximity of green areas on respiratory symptoms in children. A pan-European study within the HEALS project”. Brescianini S, Parmes E, Pärkkä J, Maesano C, Sabel C., Baldacci S, Maio S, Calamandrei G, Pesce G, Tratnik J, Medda E, Marchetti P, Panunzi S, Bono R, **Squillacioti G**, et al. May 2019. https://cris.vtt.fi/en/publications/influence-of-the-proximity-of-green-areas-on-respiratory-symptoms. The *2nd Annual UNIBS/ISMMS Collaborative Conference Exposome Symposium, Brescia 2019*
10. “Is the association between physical activity and lung function causal? An application of structural equation modeling and marginal structural modeling”. Bédard A, Accordini S, Carsin AE, Dharmage S, Fuertes E, Garcia-Larsen V, Heinrich J, Janson C, Jarvis D, Johannessen A, Leynaert B, Antonio Maldonado Pérez J, Peralta G, Pin I, **Squillacioti G.**, Weyler J, Garcia-Aymerich J. doi: 10.1183/13993003.congress-2019.PA2801. The *6th Barcellona-Boston Lung Conference, Barcelona 2019*
11. “The effect of vigorous physical activity on adult asthma incidence over 10 years” – Russell M, Dharmage S, Fuertes E, Abramson M, Bono R, Carsin AE, Emtner M, Pascual Erquicia S, Jarvis D, Johannessen A, Heinrich J, Garcia-Larsen V, Leynaert B, Marcon A, Raherison C, Somar J, **Squillacioti G**, Garcia-Aymerich J. European Respiratory Journal 2018 52: PA3993; doi: 10.1183 / 13993003.congress-2018.PA3993. The *28th International Congress of the European Respiratory Society (ERS), Paris 2018*
12. “Any correlation between the results of skin-prick test and the severity of asthma?” – Pizzimenti S, Piccioni P, Bugiani M, Carosso A, Trucco G, Tassinari R, **Squillacioti G**, Corsico A, Verlato G, Bono R. European Respiratory Journal 2017 50: PA4041; doi: 10.1183/1393003.congress-2017.PA4041. The *27th International Congress of the European Respiratory Society (ERS), Milan 2017*

13. "L'ANDAMENTO DELLO STRESS OSSIDATIVO IN ETA' ADOLESCENZIALE COME CONSEGUENZA DELLA ESPOSIZIONE A BPA" Tassinari R., Bellisario V., Occhipinti A., **Squillacioti G.**, Bugiani M., Bono R. The *50th National Congress of the Società Italiana di Igiene, Medicina Preventiva e Sanità Pubblica (SItI), Turin 2017*
14. "IL RUOLO DELLE POLVERI DI LEGNO E DELLA FORMALDEIDE NELL'INDUZIONE DI STRESS OSSIDATIVO IN UNA POPOLAZIONE DI LAVORATORI ESPOSTI" Trucco G., Ghelli F., **Squillacioti G.**, Grignani E., Negri S., Quaglio F., Bugiani M., Bono R. The *50th National Congress of the Società Italiana di Igiene, Medicina Preventiva e Sanità Pubblica (SItI), Turin 2017*

Annex II – Search strings used in the systematic review search strategy

Pubmed

("Oxidative Stress"[Mesh] OR "Antioxidants"[Mesh] OR oxidative[tiab] OR oxidation[tiab] OR oxidant[tiab] OR anti-oxidant[tiab] OR antioxidant[tiab] OR antioxidative[tiab] OR anti-oxidative[tiab] OR "Malondialdehyde"[Mesh] OR malondialdehyde[tiab] OR malonylaldehyde[tiab] OR malonaldehyde[tiab] OR malonyldialdehyde[tiab] OR MDA[tiab] OR "8-Hydroxy-2'-Deoxyguanosine"[Mesh] OR 8-hydroxy-2'-deoxyguanosine[tiab] OR 8-hydroxy-deoxyguanosine[tiab] OR 8-hydroxydeoxyguanosine[tiab] OR 8-hydroxyguanine[tiab] OR 8-hydroxy-guanine[tiab] OR 8-Oxo-2'-Deoxyguanosine[tiab] OR 8-Oxo-Deoxyguanosine[tiab] OR 8-oxo-dGuo[tiab] OR 8-Ohdg[tiab] OR 8OHdG[tiab] OR 8-OH-dG[tiab] OR 8-ohg[tiab] OR 8-hydroxy-g[tiab] OR 8-hydroxy-dg[tiab] OR 8-oxodG[tiab] OR 8-oxodGuo[tiab] OR 8-oxo-dG[tiab] OR 8-OH-2dG*[tiab] OR 8-isoprostane*[tiab] OR "F2-Isoprostanes"[Mesh] OR IsoP[tiab] OR F2-isoprostane*[tiab] OR "Dinoprost"[Mesh] OR dinoprost[tiab] OR 15-f2t-isop[tiab] OR 8-iso-PGF2a[tiab] OR 8-isoprostaglandin-f2[tiab] OR 8-iso-prostaglandin-f2[tiab] OR 8-iso-PGF2a[tiab] OR 8-epi-prostaglandin-F2alpha[tiab] OR 8-epi-prostaglandin-f2alpha[tiab] OR 8-epiprostaglandin-f2alpha[tiab] OR 8-epi-PGF2alpha[tiab] OR "Allantoin"[Mesh] OR allantoin*[tiab] OR 2,5-dioxo-4-imidazolidinyl*[tiab] OR glyoxyldiureide*[tiab] OR 5-ureidohydantoin*[tiab] OR total-antioxidant-capacity[tiab] OR total-anti-oxidant-capacity[tiab] OR total-antioxidant-power[tiab] OR total-anti-oxidant-power[tiab] OR "Thiobarbituric Acid Reactive Substances"[Mesh] OR TBARS[tiab] OR thiobarbituric-acid-reactive-substance*[tiab] OR "Glutathione"[Mesh] OR "Glutathione Peroxidase"[Mesh] OR glutathion*[tiab] OR GSH[tiab] OR GSSH[tiab] OR GSH/GSSG[tiab] OR GPX[tiab] OR "Uric Acid"[Mesh] OR uric-acid[tiab] OR UA[tiab] OR "Superoxide Dismutase"[Mesh] OR dismutase*[tiab] OR SOD[tiab] OR "Lipid Peroxides"[Mesh] OR lipid-peroxid*[tiab] OR hydroperoxid*[tiab] OR lipoperoxid*[tiab] OR "Advanced Oxidation Protein Products"[Mesh] OR AOPPs[tiab] OR "Glycation End Products, Advanced"[Mesh] OR glycation-endproduct*[tiab] OR glycation-end-product*[tiab] OR maillard*[tiab] OR "dityrosine"[Supplementary Concept] OR dityrosin*[tiab] OR bityrosin*[tiab] OR "4-oxo-2-nonenal"[Supplementary Concept] OR 4-oxo-2-nonenal*[tiab] OR 4-oxonon-2-enal*[tiab] OR 4-ONE[tiab] OR "Acrolein"[Mesh] OR acrolein*[tiab] OR acraldehyd*[tiab] OR acrylic-aldehyd*[tiab] OR 2-propenal*[tiab] OR "4-hydroxy-2-nonenal"[Supplementary Concept] OR 4-hydroxy-2-nonenal[tiab] OR 4-hydroxynonen-2-al[tiab] OR 4-HNE[tiab] OR 4-hydroxynonenal[tiab]) AND ("Exercise"[Mesh] OR "Physical Exertion"[Mesh] OR "Physical Functional Performance"[Mesh] OR "Sports"[Mesh] OR "Athletes"[Mesh] OR "Leisure Activities"[Mesh] OR (physical[tiab] AND (activit*[tiab] OR exertion[tiab])) OR exercise*[tiab] OR training[tiab] OR fitness[tiab] OR endurance[tiab] OR sport*[tiab] OR gymn*[tiab] OR running[tiab] OR runner*[tiab] OR athlet*[tiab] OR marathon*[tiab] OR jogg*[tiab] OR swimm*[tiab] OR walking[tiab] OR walker*[tiab] OR leisure*[tiab] OR treadmill*[tiab] OR bicycl*[tiab] OR volley*[tiab] OR soccer*[tiab] OR football*[tiab]) AND (urine[subheading] OR "Urine"[Mesh] OR "Urinalysis"[Mesh] OR urine[tiab] OR urines[tiab] OR urinary[tiab] OR urinalys*[tiab] OR "Saliva"[Mesh] OR saliva*[tiab] OR oral-fluid*[tiab] OR non-invasive*[tiab] OR non-intrusive*[tiab] OR noninvasive*[tiab] OR nonintrusive*[tiab] OR micro-invasive*[tiab] OR microinvasive*[tiab])

EMBASE

('oxidative stress'/exp OR 'antioxidant'/exp OR oxidative:ti,ab,kw OR oxidation:ti,ab,kw OR oxidant:ti,ab,kw OR anti-oxidant:ti,ab,kw OR antioxidant:ti,ab,kw OR antioxidative:ti,ab,kw OR anti-oxidative:ti,ab,kw OR 'malonaldehyde'/exp OR malondialdehyde:ti,ab,kw OR malonylaldehyde:ti,ab,kw OR malonaldehyde:ti,ab,kw OR malonyldialdehyde:ti,ab,kw OR MDA:ti,ab,kw OR '8 hydroxydeoxyguanosine'/exp OR 8-hydroxy-2-deoxyguanosine:ti,ab,kw OR 8-hydroxy-deoxyguanosine:ti,ab,kw OR 8-hydroxydeoxyguanosine:ti,ab,kw OR 8-hydroxyguanine:ti,ab,kw OR 8-hydroxy-guanine:ti,ab,kw OR 8-Oxo-2-Deoxyguanosine:ti,ab,kw OR 8-Oxo-Deoxyguanosine:ti,ab,kw OR 8-oxo-dGuo:ti,ab,kw OR 8-Ohdg:ti,ab,kw OR 8OHdG:ti,ab,kw OR 8-OH-dG:ti,ab,kw OR 8-ohg:ti,ab,kw OR 8-hydroxy-g:ti,ab,kw OR 8-hydroxy-dg:ti,ab,kw OR 8-oxodG:ti,ab,kw OR 8-oxodGuo:ti,ab,kw OR 8-oxo-dG:ti,ab,kw OR 8-OH-2dG*:ti,ab,kw OR 8-isoprostane*:ti,ab,kw OR 'isoprostane derivative'/exp OR IsoP:ti,ab,kw OR F2-isoprostane*:ti,ab,kw OR 'prostaglandin E2'/exp OR dinoprost:ti,ab,kw OR 15-f2t-isop:ti,ab,kw OR 8-iso-PGF2a:ti,ab,kw OR 8-isoprostaglandin-f2:ti,ab,kw OR 8-iso-prostaglandin-f2:ti,ab,kw OR 8-iso-PGF2a:ti,ab,kw OR 8-epi-prostaglandin-F2alpha:ti,ab,kw OR 8-epi-prostaglandin-f2alpha:ti,ab,kw OR 8-epiprostaglandin-f2alpha:ti,ab,kw OR 8-epi-PGF2alpha:ti,ab,kw OR 'allantoin'/exp OR allantoin*:ti,ab,kw OR dioxo-4-imidazolidinyl*:ti,ab,kw OR glyoxyldiureide*:ti,ab,kw OR 5-ureidohydantoin*:ti,ab,kw OR total-antioxidant-capacity:ti,ab,kw OR total-anti-oxidant-capacity:ti,ab,kw OR total-antioxidant-power:ti,ab,kw OR total-anti-oxidant-power:ti,ab,kw OR 'thiobarbituric acid reactive substance'/exp OR TBARS:ti,ab,kw OR thiobarbituric-acid-reactive-substance*:ti,ab,kw OR 'glutathione'/exp OR 'glutathione peroxidase'/exp OR glutathion*:ti,ab,kw OR GSH:ti,ab,kw OR GSSH:ti,ab,kw OR GSSG:ti,ab,kw OR GPX:ti,ab,kw OR 'uric acid'/exp OR uric-acid:ti,ab,kw OR UA:ti,ab,kw OR 'superoxide dismutase'/exp OR dismutase*:ti,ab,kw OR SOD:ti,ab,kw OR 'lipid peroxide'/exp OR lipid-peroxid*:ti,ab,kw OR hydroperoxid*:ti,ab,kw OR lipoperoxid*:ti,ab,kw OR 'advanced oxidation protein product'/exp OR AOPPs:ti,ab,kw OR 'advanced glycation end product'/exp OR glycation-endproduct*:ti,ab,kw OR glycation-end-product*:ti,ab,kw OR maillard*:ti,ab,kw OR dityrosin*:ti,ab,kw OR bityrosin*:ti,ab,kw OR 4-oxo-2-nonenal*:ti,ab,kw OR 4-oxonon-2-enal*:ti,ab,kw OR 4-ONE:ti,ab,kw OR 'acrolein'/exp OR acrolein*:ti,ab,kw OR acraldehyd*:ti,ab,kw OR acrylic-aldehyd*:ti,ab,kw OR 2-propenal*:ti,ab,kw OR '4 hydroxynonenal'/exp OR 4-hydroxy-2-nonenal:ti,ab,kw OR 4-hydroxynonen-2-al:ti,ab,kw OR 4-HNE:ti,ab,kw OR 4-hydroxynonenal:ti,ab,kw) AND ('physical activity, capacity and performance'/exp OR 'sport'/exp OR 'athlete'/exp OR 'recreation'/exp OR (physical:ti,ab,kw AND (activit*:ti,ab,kw OR exertion:ti,ab,kw)) OR exercise*:ti,ab,kw OR training:ti,ab,kw OR fitness:ti,ab,kw OR endurance:ti,ab,kw OR sport*:ti,ab,kw OR gymn*:ti,ab,kw OR running:ti,ab,kw OR runner*:ti,ab,kw OR athlet*:ti,ab,kw OR marathon*:ti,ab,kw OR jogg*:ti,ab,kw OR swimm*:ti,ab,kw OR walking:ti,ab,kw OR walker*:ti,ab,kw OR leisure*:ti,ab,kw OR treadmill*:ti,ab,kw OR bicycl*:ti,ab,kw OR volley*:ti,ab,kw OR soccer*:ti,ab,kw OR football*:ti,ab,kw) AND ('urine'/exp OR 'urinalysis'/exp OR urine:ti,ab,kw OR urines:ti,ab,kw OR urinary:ti,ab,kw OR urinalys*:ti,ab,kw OR 'saliva'/exp OR saliva*:ti,ab,kw OR oral-fluid*:ti,ab,kw OR non-invasive*:ti,ab,kw OR non-intrusive*:ti,ab,kw OR noninvasive*:ti,ab,kw OR nonintrusive*:ti,ab,kw OR micro-invasive*:ti,ab,kw OR microinvasive*:ti,ab,kw)

Cochrane CENTRAL

#1 MeSH descriptor: [Oxidative Stress] explode all trees
#2 MeSH descriptor: [Antioxidants] explode all trees
#3 (oxidative OR oxidation OR oxidant OR anti-oxidant OR antioxidant OR antioxidative OR anti- oxidative):ti,ab,kw
#4 MeSH descriptor: [Malondialdehyde] explode all trees
#5 (malondialdehyde OR malonylaldehyde OR malonaldehyde OR malonyldialdehyde OR MDA):ti,ab,kw
#6 MeSH descriptor: [8-Hydroxy-2'-Deoxyguanosine] explode all trees
#7 ("8-hydroxy-2'-deoxyguanosine" OR "8-hydroxy-deoxyguanosine" OR "8-hydroxydeoxyguanosine" OR "8-hydroxyguanine" OR "8-hydroxy-guanine" OR "8-Oxo-2'-Deoxyguanosine"):ti,ab,kw
#8 ("8-Oxo-Deoxyguanosine" OR "8-oxo-dGuo" OR "8-Ohdg" OR 8OHdG OR "8-OH-dG" OR "8-ohg" OR "8-hydroxy-g" OR "8-hydroxy-dg" OR "8-oxodG" OR "8-oxodGuo" OR "8-oxo-dG" OR "8-OH-2dG" OR "8-isoprostane"):ti,ab,kw
#9 MeSH descriptor: [F2-Isoprostanes] explode all trees
#10 (IsoP OR "F2-isoprostane"):ti,ab,kw
#11 MeSH descriptor: [Dinoprost] explode all trees
#12 (dinoprost OR "15-f2t-isop" OR "8-iso-PGF2a" OR "8-isoprostaglandin-f2" OR "8-iso-prostaglandin-f2" OR "8-iso-PGF2a" OR "8-epi-prostaglandin-F2alpha" OR "8-epi-prostaglandin-f2alpha" OR "8-epiprostaglandin-f2alpha" OR "8-epi-PGF2alpha"):ti,ab,kw
#13 MeSH descriptor: [Allantoin] explode all trees
#14 (allantoin* OR "2,5-dioxo-4-imidazolidinyl" OR glyoxyldiureide OR "5-ureidohydantoin" OR "total antioxidant capacity" OR "total anti-oxidant capacity" OR "total antioxidant power" OR "total anti- oxidant power"):ti,ab,kw
#15 MeSH descriptor: [Thiobarbituric Acid Reactive Substances] explode all trees
#16 (TBARS OR "thiobarbituric acid reactive substances"):ti,ab,kw
#17 MeSH descriptor: [Glutathione] explode all trees
#18 (glutathion* OR GSH OR GSSH OR "GSH/GSSG" OR GPX):ti,ab,kw
#19 MeSH descriptor: [Uric Acid] explode all trees
#20 ("uric acid" OR UA):ti,ab,kw
#21 MeSH descriptor: [Superoxide Dismutase] explode all trees
#22 (dismutase* OR SOD):ti,ab,kw
#23 MeSH descriptor: [Lipid Peroxidation] explode all trees
#24 ("lipid peroxidation" OR hydroperoxid* OR lipoperoxid*):ti,ab,kw
#25 MeSH descriptor: [Advanced Oxidation Protein Products] explode all trees
#26 (AOPPs):ti,ab,kw
#27 MeSH descriptor: [Glycation End Products, Advanced] explode all trees
#28 ("glycation endproducts" OR "glycation end-products" OR maillard* OR dityrosin* OR bityrosin* OR "4-oxo-2-nonenal" OR "4-oxonon-2-enal" OR "4-ONE"):ti,ab,kw
#29 MeSH descriptor: [Acrolein] explode all trees
#30 (acrolein* OR acraldehyde* OR "acrylic aldehyde" OR "2-propenal" OR "4-hydroxy-2-nonenal" OR "4-hydroxynonen-2-al" OR "4-HNE" OR "4-hydroxynonenal"):ti,ab,kw
#31 #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25 OR #26 OR #27 OR #28 OR #29 OR #30
#32 MeSH descriptor: [Exercise] explode all trees
#33 MeSH descriptor: [Physical Exertion] explode all trees
#34 MeSH descriptor: [Physical Functional Performance] explode all trees
#35 MeSH descriptor: [Sports] explode all trees
#36 MeSH descriptor: [Athletes] explode all trees
#37 MeSH descriptor: [Leisure Activities] explode all trees
#38 ((physical AND (activit* OR exertion))):ti,ab,kw
#39 (exercise* OR training OR fitness OR endurance OR sport* OR gymn* OR running OR runner* OR athlet* OR marathon* OR jogg* OR swimm* OR walking OR walker* OR leisure* OR treadmill* OR bicycl* OR volley* OR soccer* OR football*):ti,ab,kw
#40 #32 OR #33 OR #34 OR #35 OR #36 OR #37 OR #38 OR #39
#41 MeSH descriptor: [Urine] explode all trees
#42 MeSH descriptor: [Urinalysis] explode all trees
#43 (urine OR urines OR urinary OR urinalys*):ti,ab,kw
#44 MeSH descriptor: [Saliva] explode all trees
#45 (saliva* OR "oral fluid" OR "oral fluids" OR non-invasive* OR non-intrusive* OR noninvasive* OR nonintrusive* OR micro-invasive* OR microinvasive*):ti,ab,kw
#46 #41 OR #42 OR #43 OR #44 OR #45

Annex III – Scientific papers *in extenso*

Scientific papers, that have been already published, will be attached in the following pages.

International Journal of
Environmental Research and Public Health

MDPI

Article

The Asti Study: The Induction of Oxidative Stress in A Population of Children According to Their Body Composition and Passive Tobacco Smoking Exposure

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Abstract: Obesity and exposure to second-hand tobacco smoking (SHS) may influence oxidative stress (OS) levels, especially in children. This study investigated body composition and SHS influence on OS induction in the paediatric population. The first purpose was identifying an appropriate BMI standard for adiposity assessment in OS investigations. Secondly, SHS and obesity were analysed as inductors of OS. The epidemiologic sample involved 330 children. Three BMI (body mass index) references (IOTF, CDC, and WHO) and an impedentiometric scale supplied body-composition measurements. Partecipants filled out a questionnaire and provided urinary samples for biomarker quantifications: isoprostane (15-F2t IsoP) and cotinine as OS and SHS biomarker, respectively. Obesity prevalence changed over different BMI references (14%, 21%, and 34% for IOTF, CDC, and WHO, respectively). Obese children, by IOTF, showed an increase of 56% in 15-F2t IsoP compared to those normal weight ($p = 0.020$). Children belonging to the third and the fourth cotinine quartile compared to those of the first quartile had higher 15-F2t IsoP (1.45 ng/mg, 95% CI: 1.06–1.97, $p = 0.020$ and 2.04 ng/mg, 95% CI: 1.55–2.69, $p < 0.0001$, respectively). Obesity assessment in children requires appropriate BMI reference depending on research field. Both SHS exposure and obesity may increase OS in children.

Keywords: oxidative stress; children; Public Health; obesity; BMI; second-hand smoke

1. Introduction

Inflammation and oxidative stress (OS) are often considered as pre-pathological conditions originated from numerous non-optimal environments and working circumstances [1], from modifiable habits (e.g., tobacco smoking) [2,3] and from several health impairments which may represent both their cause and effect [4]. Various risk factors are able to induce redox imbalance in humans, and among these, adiposity [5,6] and second hand smoke (SHS) may play an important role in OS induction, in particular in youths [7–9]. Indeed, excessive adipose tissue and high exposure to SHS may induce a dysregulation of the balance between oxidants and antioxidants in favor of the oxidants [10,11], leading to OS [12], which determines a condition that often precedes the onset of several diseases [13,14]. Active tobacco smoking is able to promote oxidant production and to deplete the antioxidant defences of the body, and cigarette smoking has been related to a statistically significant decrease of the total antioxidant status, thus it may have a role in the mechanism by which tobacco smoking promotes inflammation [15]. SHS, to which more than 40% of children below 15 years of age are exposed [16],

is associated with higher incidence of numerous health problems, including infections, asthma, and the decrease of lung function [3,17]. Moreover, free radicals may damage organs and tissues, playing a central role in passive-smoking-mediated disorders [9]. Environmental or household pollution can lead to increased severity and exacerbation of inflammatory diseases in children [18,19], who are the most vulnerable portion of the population as their respiratory, immune, and nervous systems are still in development [20]. Other factors, such as diet, qualitatively and quantitatively unsuitability, and scarce physical activity (PA), may adversely influence body composition, leading to overweight or obesity, which represent emerging issues worldwide, especially in the paediatric population [21,22]. The prevalence of overweight and obesity among children and adolescents aged 5–19 has risen from 4% in 1975 to over 18% in 2016 [23]. Childhood obesity is associated with higher future risks of obesity, premature death, and disability in adulthood. The excess of adipose tissue has been identified as a source of pro-inflammatory cytokines [24], which generate, in turn, further reactive oxygen species, "ROS", in tissues, increasing the lipid peroxidation rate [25]. In obese subjects, adipose tissue determines an increase both in local and systemic production of pro-inflammatory adipocytokines that, in turn, induce OS [26]. Furthermore, OS promotes a systematic and systemic low-grade inflammatory response [27,28]. Inflammation of adipose tissue plays a critical role in the pathogenesis of obesity-related complications and several other diseases all associated with OS [28,29]. The Asti Study takes its name from the city of Piedmont, a region in the northwest part of Italy, where the subjects were recruited. It was designed to attain, in preventative terms, some of the essential objectives of Public Health in the paediatric population. In particular, this cross-sectional study aims to evaluate, in a large group of school-age children living in Asti, the role of body composition and SHS, as possible health risk factors able to influence the OS levels. The Asti study protocol provides details concerning the enrolment phases, the structuring of the questionnaire, the sampling of urine, and the biological and statistical analyses. OS has been evaluated by means of quantification of urinary isoprostane (15-F_{2t} IsoP), a prostaglandin-like compound derived from lipid peroxidation of arachidonic acid, widely used as an in vivo OS biomarker. Body composition was assessed by Body Mass Index (BMI) and Body Fat percentage (BF %). Moreover, to establish the most appropriate BMI standard in defining obesity condition of children in OS investigations, three different international references were selected and compared.

2. Materials and Methods

2.1. Study Area

Asti is a town of 76.211 inhabitants, 503 per km^2, (01/01/2018 - ISTAT) located 123 meters above sea level in the Piedmont region, 55 km from Turin, the capital of Piedmont, the westernmost Region of the Po Valley. The Piedmont region, as well as the entire Padana Valley, is known for its high levels of atmospheric pollution due to the orographic position, the mountain range of the Alps, and the meteorological conditions, which led to the stagnation of the air masses reducing, at the same time, the dilution of pollutants. Based on these environmental characteristics, this specific study area deserves to be deeply investigated with regard to the relationship between airborne pollutants, OS, and respiratory health [30].

2.2. Ethics Committee Approval

All subjects gave their informed consent for inclusion before enrolment to the study. The study was conducted in accordance with the Declaration of Helsinki, and the Ethics Committee of Comitato Etico Internazionale A.O.U. Luigi Gonzaga (no. 0005540, tit. approved the protocol II, cat. 02, Cl. 01).

2.3. Selection of Subjects

The ASTI study is based on a population recruited from five primary schools located within the municipal boundaries of the city of Asti. All subjects participated as volunteers and they were enrolled according to the following inclusion criteria: only healthy children ranging from 8 to 11 years of age by

March 2017, and resident in the selected area. Parents or legal tutors of each participant were asked to sign an informed consent and fill out a self-administered questionnaire. Then, each subject underwent the measurement of body composition and provided a urine sample for the quantification of: 15-F_{2t} IsoP, to quantify OS, and cotinine (COT) as biomarker of tobacco smoking exposure.

2.4. Questionnaire

The questionnaire was prepared according to the SIDRIA questionnaire [7,31] and was addressed and self-administered to the parents of the young volunteers. The questionnaire includes general information, address and residential area, house features, respiratory symptoms and allergies, family and socioeconomic status, SHS exposure, nutrition, physical activity, and physical inactivity.

2.5. Height

Height was estimated using a stadiometer (GIMA professional medical products) positioned on the wall at 2 meters of height in line with the surface base.

2.6. Impedance

Measurements of body composition were performed using an impedentiometric scale (FitScan BC-545F Tanita®), which adopts advanced Bioelectric Impedance Analysis (BIA) technology. Each subject was measured wearing light clothes and without shoes and socks. A skilled operator guided the subject onto the scale and recorded all data, expressed with decimal accuracy. For children (aged 7–17), the scale only displays three parameters, namely Body weight (kg), BMI (Kg/m^2), and body fat (%) (BF). Two-compartment models, such as BIA, are capable to discern fat mass (FM) from fat free mass (FFM) in order to divide the total body mass into FM + FFM. We calculated FM index (FMI) expressed as kg/m^2 from the ratio of BF to height squared and obtained FFM index (FFMI) as a complementary measure of FMI in BMI calculation.

2.7. BMI

To categorise subjects according to their body weight, BMI was used as measure of body composition. It was categorised according to three different classification systems proposed by the Centers for Disease Control and Prevention "CDC" [32], the International Obesity Task Force "IOTF" [33,34], and the World Health Organization "WHO" [35], respectively, and all cut-off values were sex and age-adjusted. Underweight (UW), normal weight (NW), overweight (OW), and obese (OB) categories were defined as (i) extrapolation of the adult BMI cut-off points according to the IOTF standard, (ii) based on centiles, in the case of the CDC reference, and (iii) according to standard deviations from the mean values of the WHO reference.

2.8. Urine

A spot of fresh urine was collected from each volunteer, aliquoted, and stored at −80 °C until analysis, performed within 2 months.

2.8.1. Urinary 15-F_{2t} IsoP

The 15-F_{2t} IsoP was measured as biomarker of OS by E.L.I.S.A. technique according to manufacturer's instructions (Oxford, MI, USA). The declared limit of detection is 0.2 ng/mL. Dilution 1:4 was made to achieve better assay accuracy. A preliminary incubation with β-glucuronidase for 2 h at 37 °C was performed to detect the entire quantity of 15-F_{2t} IsoP present in each urine sample, mostly excreted in human urine as glucuronic acid conjugated (over 50%) [36,37].

2.8.2. Urinary Cotinine

COT was quantified as biomarker of tobacco smoking exposure as follows: transferring 10 mL of urine into a glass tube and adding 4.25 g of NaCl, 500 µL of NaOH (5 M), and 10 µL of cotinine-d3

as internal standard. Adding 2 mL of trichloromethane ($CHCl_3$) to perform a double liquid–liquid extraction. Centrifuging the sample for 10 minutes at 3000 rpm and collecting the resulting organic phase a new glass tube; evaporating to dryness under a gentle steam of N_2. The dry residue was reconstituted in 200 µL of $CHCl_3$ and transferred into a conical vial for GC-MS determination. More details are described in previous studies [38].

2.8.3. Urinary Creatinine

Creatinine was determined by the kinetic Jaffé procedure to normalise the excretion rate of all urinary biomarkers measured: 15-F_{2t} IsoP and COT.

2.9. Statistical Analyses

Descriptive analyses and comparison of socioeconomic characteristics, SHS, and body composition by gender and obese *versus* normal weight BMI classifications were carried out with the chi-square test for categorical variables (ethnicity and age classes), and the t test or Mann–Whitney U-test for quantitative parameters, as appropriate. The relationship between 15-F_{2t} IsoP, as a continuous dependent variable, and BMI classification according to IOTF, as an independent variable (NW as the reference category) was evaluated by Log-link Gaussian Generalised Linear Model (GLM), adjusted for cotinine quartiles, physical activity, gender, age, and body fat. Linear regression analyses were used to compare the relationship between BMI, as dependent variable and FMI, as independent variable, and the relationship between BMI as dependent variable and FFMI, as independent variable. All the tests were two-tailed and the level of significance was set at 0.05. All analyses were performed using STATA SE v14.2 (Stata Corp, College Station, TX, USA).

3. Results

The epidemiologic sample of this study consists of 330 children aged between 8 and 11 years. The description of the sample is provided in Table 1, which shows that the sample is homogeneous by gender, age, and ethnicity. Males and females were also compared with each other for height, weight, and body composition (described by BMI as continue variables, BF %, and FMI-FFMI body components).

Table 1. Descriptive and physical characteristics of the epidemiologic sample.

		Females *n* = 161 (48.8%)	**Males *n* = 169 (51.2%)**	***p*-Value**	**Total 330**
Age (years)	8	51 (31.7)	56 (33.1)	0.84	107 (32.4)
	9	58 (36.0)	53 (31.4)		111 (33.6)
	10+	52 (32.3)	60 (35.5)		112 (33.9)
Ethnicity (n)	Non-Caucasian mothers [a]	15 (9.3)	17 (10.6)	0.32	32 (9.7)
	Non-Caucasian fathers [a]	17 (10.1)	17 (10.1)	0.54	34 (10.3)
Height (cm)		138.4 ± 9.3	138.9 ± 8.4	0.57	138.4 ± 8.7
Weight (kg)		36.5 ± 10.1	36.8 ± 10.8	0.78	36.3 ± 10.2
BMI (kg/m^2)		19.1 ± 3.6	18.8 ± 3.6	0.28	18.8 ± 0.2
FMI (kg/m^2)		5.2 ± 2.2	4.8 ± 2.3	0.07	5.0 ± 2.3
FFMI (kg/m^2)		13.9 ± 1.9	14.0 ± 1.6	0.44	14.0 ± 1.8
Body Fat (%)		26.9 ± 6.2	24.3 ± 6.6	<0.0001	25.4 ± 6.5

Notes: Height, weight BMI, Body Fat %, FMI and FFMI are expressed as mean ± SD. [a] Non-Caucasian includes African, Asiatic, and Hispanic ethnicities.

Only BF % is significantly higher ($p < 0.0001$) in females. Figure 1 shows children categorisations based on three selected international BMI standards and referred to their principal sub-groups: NW, OW, and OB. Underweight group was less than 5% of the whole sample in each BMI standard, thus it has been considered together with the NW group. The prevalence of obesity varies using different BMI standards—WHO reports the highest numerousness of obese children, CDC categorises almost equally

OW and OB, and IOTF shows the lowest prevalence of obesity. Moreover, 15-F_{2t} IsoP distribution does not change over UW+NW, OW, and OB categories, whereas there is a tendency of 15-F_{2t} IsoP increase from UW+NW to OB category. Levels of 15-F_{2t} IsoP between IOTF standard categories are equal to 4.5 ± 3.8, 4.9 ± 3.9, and 5.7 ± 4.7 ng/mg Crea in UW+NW, OW, and OB, respectively (p = 0.091). According to CDC categorisations, OS biomarker levels are 4.3 ± 3.8, 4.8 ± 3.4, and 5.3 ± 4.7 ng/mg Crea in UW+NW, OW, and OB children (p = 0.356). The WHO categories of UW+NW, OW, and OB correspond to 4.4 ± 3.9, 5.0 ± 3.3, and 5.1 ± 4.3 ng/mg Crea of 15-F_{2t} IsoP, respectively (p = 0.216). Although no difference in 15-F_{2t} IsoP distribution is significant among UW+NW, OW, OB groups, the IOTF standard shows the nearest significance level, reporting a p-value of 0.091.

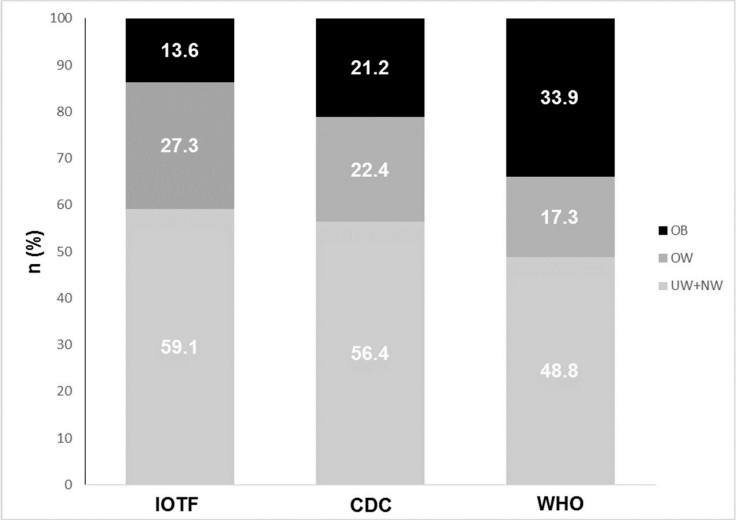

Figure 1. Prevalence of normal weight "NW", over weight "OW", and obese "OB" children categorised by three different BMI references: IOTF, CDC, and WHO.

Table 2 reports the results of the Generalised Linear Model (GLM), adjusted by SHS as confounder, sex, age, BF %, and PA. Both being obese by IOTF standard and exposed to SHS determines an increase in urinary 15-F_{2t} IsoP.

Table 2. Generalised linear model with 15-F_{2t} IsoP as dependent variable, fully adjusted for sex, age, body fat percentage, physical activity, BMI categories by IOTF, and cotinine quartiles.

15-F_{2t} IsoP	Exp (β) (95% C.I.)	p-Value
Body composition [a]:		
Overweight (IOTF)	1.22 (0.97–1.56)	0.095
Obese (IOTF)	1.56 (1.07–2.27)	0.020
Cotinine quartiles [b]:		
COT 2nd quartile	1.27 (0.93–1.72)	0.130
COT 3rd quartile	1.45 (1.06–1.97)	0.020
COT 4th quartile	2.04 (1.55–2.69)	<0.0001
Physical activity [c]:		
Moderate	1.00 (0.83–1.23)	0.944
Intense	1.14 (0.81–(1.61)	0.440
General characteristics [d]:		
Sex	1.09 (0.92–1.31)	0.297
Age	1.06 (0.96–1.15)	0.210
Body fat (%)	1.00 (0.97–1.01)	0.110

[a] Body composition expressed through BMI categories by IOTF cut-offs (UW+NW as reference group. [b] Cotinine: 1st quartile as reference. [c] Physical activity: up to one day/week as reference, "moderate" = 2–4 days/week and "intense" = 5–7 days/week. [d] sex: female as reference gender; BF%: as continue variable.

In order to establish whether BMI was a reliable proxy of obesity status in our sample, we also analysed BMI correlations and increments with respect to contributions of the two body-composition compartments: FMI and FFMI. As shown in Figure 2, BMI is significantly and positively correlated to both FMI and FFMI ($p < 0.0001$). Furthermore, in order to calculate BMI gradients, a subgroup of children ($n = 90$), who have been categorised OB and NW by all the three standards simultaneously, were selected from the whole sample. A number of 45 obese subjects was considered as reference to randomly select a homogeneous sample, in terms of sex and age, of 45 other children categorised as NW by all the three standards.

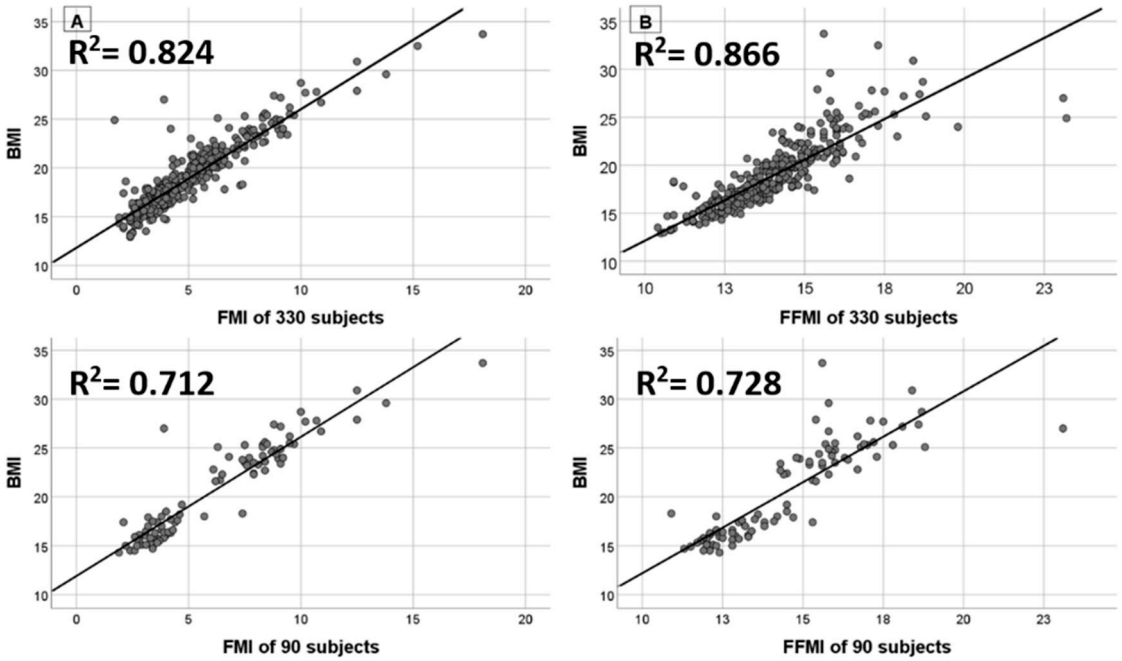

Figure 2. Linear regression between BMI and FMI on the left (**A**), and BMI and FFMI on the right (**B**). The upper-side scatterplots refer to the whole sample (n = 330) and the lower-side scatterplots refer to the subgroup of children categorised in accordance with all BMI standards simultaneously (n = 90).

Table 3 shows as FMI and FFMI mean values are significantly higher in OB children than in those who are NW ($p < 0.0001$). Moreover, 15-F_{2t} IsoP displays significant differences between two groups, with higher levels in OB subjects, for both Ln-transformed values and not-transformed, $p = 0.002$ and $p = 0.038$, respectively. Taking into account FMI and FFMI average differences in OB and NW children, we calculated gradients to assess whether BMI higher levels were mostly due to an average increase of FMI, thus adiposity, or on the contrary, a greater average raise in the amount of FFMI and to support results displayed in Figure 2. FMI and FFMI mean values of the gradients are statistically different and in particular FMI has the highest gradient mean value equal to 5.2 ± 2.5 Kg/m_2 compared to FFMI gradient mean value of 3.5 ± 1.9 Kg/ m^2 ($p < 0.0001$).

Table 3. Subgroup of children categorised as NW and OB by all standards simultaneously.

	Normal Weight $n = 45$	Obese $n = 45$	p-Value	Gradients Δ
AGE (years)	9.2 ± 0.9	9.2 ± 1.0	0.81	
FMI (Kg/m^2)	3.6 ± 0.9	8.8 ± 2.3	<0.0001	5.2 ± 2.5
FFMI (Kg/m^2)	12.8 ± 0.9	16.3 ± 1.6	<0.0001	3.5 ± 1.9
15-F_{2t} IsoP (ng/mg Crea)	3.8 ± 3.7	5.7 ± 4.7	0.039	4.1 ± 4.8
Ln(15-F_{2t} IsoP) (ng/mg Crea)	0.99 ± 0.79	1.50 ± 0.67	0.002	0.8 ± 0.6

Values are expressed as mean ± SD.

4. Discussion

Obesity is a health worldwide issue, affecting both adults and children. This multi-factorial condition represents a risk factor for many diseases and predisposes children to chronic adulthood health problems, such as metabolic syndrome, diabetes mellitus, cardiovascular diseases, and cancer [23]. Adipose tissue has been identified as an active endocrine organ involved in the production of adipocytokines or adipokines [39]. Low-grade inflammation and OS are two of the key mechanisms implied in the obesity-induced metabolic complications [5] and in worsening obesity condition itself [28]. Moreover, excessive OS may damage cellular structures and influence the anti-oxidant defences which can be compromised and do not counteract redox disequilibrium [12].

The purpose of this study was to investigate whether body composition, and in particular, obesity condition, assessed by BMI, may influence urinary 15-F_{2t} IsoP levels and OS status in healthy children. Moreover, in order to characterise obese subjects, three international BMI references were selected, since the percentage of youths classified as overweight or obese varies considerably, depending on the BMI cut-points or centiles [40]. The three BMI standards highlighted significant differences in categorising children between categories. Indeed, the application of the IOTF standard has highlighted the smallest percentage of obese children compared to the CDC standard (14% vs 21%) and even wider difference compared to the WHO standard (14% vs 34%). Our results are in keeping with those reported by many other studies [41–43].

With regard to NW and OB children, categorised by all three standards simultaneously, overall 14% (n = 45) of the sample has been classified as obese and 44% (n = 144) were considered NW, but for the calculation of the BMI gradients, only 90 subjects (45 OB + 45 NW) were considered. By analysing the role of obesity in OS induction, this study highlights a higher effectiveness of the IOTF standard in categorising children as obese with the final purpose of investigating OS intensity. Whereas isolated analyses on BMI categorisations and OS induction do not show significant differences between groups, the fully adjusted multivariate model pinpoints that obese children, by the IOTF reference, have higher levels of urinary 15-F_{2t} IsoP. Being obese promotes an increase of 56% in 15-F_{2t} IsoP levels compared to those measured in normal weight children (p = 0.020). Interestingly, statistical analyses that took into account the other two standards did not show any results about the relationship between obesity and OS. Only IOTF seems to clarify the role of adiposity in OS induction, showing an appropriate accuracy. This result is independent from another important OS predictor: SHS exposure, which has been considered as a confounder in the GLM model. Nonetheless, SHS shows its influence on OS and children who belong to the third and the fourth COT quartile compared to those of the first quartile, who have higher 15-F_{2t} IsoP levels (1.45 ng/mg, 95% CI: 1.06–1.97, p = 0.020 and 2.04 ng/mg, 95% CI: 1.55–2.69, $p < 0.0001$, respectively).

In general, by considering SHS exposure as the independent variable in OS induction, children exposed to the highest levels of SHS exhibit higher levels of urinary 15-F_{2t} IsoP ($p < 0.0001$) and subgrouping COT in quartiles, the fourth quartile corresponds to the highest level of 15-F_{2t} IsoP. Concerning body-composition analyses, it can be noticed that BMI is positively and significantly related to both FMI and FFMI components. Interestingly, linear regression between BMI and FMI accounts for a greater value of explained variance equal to 0.824 (n = 330) and 0.866 (n = 90), which are higher than those related to BMI and FFMI regression (0.712 and 0.728 for n = 330 and n= 90, respectively). This result highlights a better fit between BMI and FMI, which in turn, reflects adiposity, and supports the usage of BMI as a proxy of obesity condition in this sample. This sub-classification also allows understanding whether BMI may be considered as a proxy of adiposity in the current study and supports its usage in body composition inquires. In this context, investigations on the relationship between FMI, FFMI, and increased BMI values highlights that higher BMIs are more influenced by an average increment of FMI than FFMI, thus the FMI component in BMI of obese children is, on average, greater than FFMI. Further analyses on the subgroup of children categorised in accordance to all three BMI standards show that OS biomarker levels are significantly different between OB children compared to those of NW. In particular, being obese, per se, implies increased levels of urinary 15-F_{2t} IsoP (p = 0.002).

In summary, both SHS and body composition, particularly obese condition, are influencing factors in OS induction. Hence, we observed that school-aged children showed higher levels of urinary 15-F_{2t} IsoP in relation to both adiposity and SHS exposure, which are independent OS predictors. In the fully adjusted multivariate model, BF %, considered as a continuous variable, did not display any influence on OS biomarker. The difference of OS observed between genders, where females show higher BF % than males ($p < 0.0001$), may be due to those girls who underwent early pubertal maturation, but no differences were observed in OS biomarkers between males and females. Future investigations could be focused on body fat profile changes over time, e.g., in a longitudinal study, where BF is considered to be more appropriate than BMI [44]. Nevertheless, our study aimed primarily to analyse BMI because of its easy measurement, cost-effectiveness, and high correlation with other tools for body composition assessment in epidemiologic studies [45]. It was valuable in establishing which BMI reference was more appropriate for OS investigations and obesity in children. Whereas all three BMI standards are effective tools in prevention strategies, their differences in accuracy, sensitivity, and specificity requires specific fields of application and IOTF appears more appropriate in OS inquiries.

Although specific fields of application deserve the most appropriate BMI reference, prevention strategies in children for public health are adequately supplied by all these BMI standards, which are valuable tools in primary prevention against obesity. Hence, all three references show their utility in defining obese children, even though they may show different sensitivity and specificity depending on the field of application. The main limitation of this study is its cross sectional design that did not allow us to explore OS and body composition changes over time and their causal relationship. Thus, only differences in obesity prevalence could be described and associated with OS induction. Furthermore, BF has been measured by the BIA method, whose prediction equation assumes 73% of water as hydration of the participants [46], which does not always reflects real sampling conditions and this aspect cannot be overcome. On the other hand, the BIA has been considered as the simplest and least expensive method for BF evaluation in clinical practice [46].

5. Conclusions

In conclusion, urinary concentration of 15-F_{2t} IsoP is significantly higher in children classified as obese by all three BMI references together as well as by IOTF standard, compared to those who were categorised as normal weight. Thus, OS is influenced by body composition, in particular by adiposity. BMI represents a suitable tool in epidemiologic study investigations on OS and a valuable instrument in screening the school-aged populations to support and promote public health, with the final purpose of defining the best prevention strategies against obesity and risky conditions for health.

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Greenness Availability and Respiratory Health in a Population of Urbanised Children in North-Western Italy

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Abstract: Paediatric Asthma contributes in paediatric global burden of diseases, as the most common chronic disease in children. Children are exposed to many environmental risk-factors, able to determine or worsen respiratory diseases, and contributing to asthma and asthma-like symptoms increases, especially in metropolitan areas. In urban settings, surrounding vegetation (greenness) may provide important benefits to health, including the promotion of physical activity and the mitigation of air and noise pollution. The aim of this study was to investigate the association between greenness and respiratory health. A total of 187 children (10–13 yrs old) were recruited in Turin, the north-western part of Italy. The prevalence of asthma and asthma-like symptoms was calculated from self-reported data collected by SIDRIA questionnaire. Spirometry test was performed to obtain respiratory flow measurements. Greenness was measured at individual level through the Normalised Difference Vegetation Index (NDVI) estimations from remote-sensing images. Higher exposure (3rd tertile vs. 1st tertile) to NDVI was associated to significantly lower ORs for asthma [0.13 CI 95% 0.02–0.7, $p = 0.019$], bronchitis [0.14 CI 95% 0.05–0.45, $p = 0.001$], and current wheezing [0.25 CI 95% 0.09–0.70, $p = 0.008$]. A significative positive association was found between greenness and $FEF_{25–75}$, since children exposed to the 2nd tertile of NDVI reported a significantly decreased $FEF_{25–75}$ compared to those in the 3rd tertile [B: −2.40; C.I.95%: −0.48–0.01; $p = 0.049$]. This cross-sectional study provided additional data on still inconsistent literature referring to respiratory health in children and green spaces, attesting a positive effect of greenness in a specific area of Italy. Further research is still needed.

Keywords: children health; greenness; respiratory function; environmental primary prevention; asthma

1. Introduction

Paediatric asthma is a widespread condition whose aetiology is multifactorial and may be influenced by a combination of individual and environmental features, such as genetic susceptibility, atopy, and several environmental exposures (e.g., traffic-related pollutants, tobacco smoking exposure, etc.) [1,2]. Asthma contributes in paediatric global burden of diseases, as the most common chronic disease in children [2]. Noticeable changes in the prevalence of asthma were described among countries [1] showing a global prevalence up to a peak of 20% in paediatric population [3]. De Marco [4] reported an increasing trend (+38%) for both asthma and asthma-like symptoms in the Italian population, from 1990 to 2010. In Italy the prevalence of asthma reached 8.6% in children aged 6–7 and 11.4% in adolescents [5]. Concerning

respiratory symptoms, current wheezing has been reported as one of the most related to asthma and its prevalence increased in both children (+11.6%) and adolescents (13.7%) [6]. In addition, the assessment of paediatric lung function is used in diagnostic evaluation of respiratory diseases [7] since lung function impairment during childhood may lead to respiratory diseases in adulthood [8] Specifically, a reduced lung functionality in children has been reported as risky for COPD overlap syndrome at 45 years old [8]. At this concern, pulmonary function tests are needful to assess respiratory status at the individual level for investigating the respiratory system and latent abnormalities [9].

Environmental conditions expose children to a variety of factors, which are able to determine or worsen respiratory or allergic diseases [10] contributing to asthma and asthma-like symptom increases, especially in metropolitan areas [11]. Furthermore, as a component of urban environment, green spaces may provide important benefits to health. Higher greenness exposure has been reported as beneficial for several health conditions, such as cardiovascular diseases, adiposity, mental health and birth outcomes [12,13]. Whereas the mechanisms behind greenness exposure and health improvements have not been totally understood yet, it may provide respiratory health improvements by mitigating air and noise pollution [14] or promoting the participation in physical activity [15]. The majority of the studies, investigating respiratory health and the environment, focused on air pollution [16–18] and, only recently, increasing attention has been paid to green spaces, whose configuration and composition may differently influence respiratory symptoms and lung function [15].

However, the existing results on greenness and respiratory outcomes are still inconsistent [19–21]. Some authors reported a positive association between urban vegetation and asthma in children [22], speculating on non-urban vegetation as a potential source for allergens [23,24], which may negatively affect respiratory health. Moreover, living close to the forests has been associated with allergic symptoms [25,26] and greenness was differently associated to allergies, also depending on the study area [27]. On the contrary, several studies stated a protective effect of greenness on respiratory diseases such as asthma. Higher greenness was associated with lower odds ratio of asthma in children with current tobacco smoking exposure and lower risk of [28]. Lovasi et al. reported an inverse association between street trees and prevalence of early childhood asthma [29]. A decreased risk of asthma was observed in children exposed to higher greenness [30], addressing to the "Biodiversity hypothesis". The proximity to residential greenness was reported as protective for bronchitis and wheezing, in the Mediterranean and Euro-Siberian region, respectively [3]. Finally, even no association has been observed [25,31–33].

Based on the existing mixed results and their dependency on the geographic area, the aim of this cross-sectional study is to investigate whether there exists an association between green spaces and respiratory health in urban settings, focusing on asthma and asthma-like symptoms in children living in northern Italy.

2. Materials and Methods

2.1. Sample Population and Ethic Committee Approval

This study is part of a research project funded by the Piedmont Regional Council focusing on the effects of environmental pollution in schoolers that started in 2002 and has followed-up in 2010. Overall, 1005 subjects were enrolled, 573 answered to the follow-up and only 223 accepted to re-perform spirometry. This cross-sectional study involved 187 out of 223 healthy children (10–13 years old) from secondary schools located in Turin, north-western part of Italy. These subjects had validated spirometry, complete questionnaires and provided their home address.

The subjects were recruited at school and participated as volunteers. Each participant gave the assent to participate and the parents or guardians signed a written informed consent to allow their children participation. All healthy volunteers, aged between 10 and 13 years old, who provided the full home address, performed a valid spirometry and reported the total completion of the respiratory questionnaire were included. The study protocol was submitted to the Ethics Committee and was carried out after its approval (Ethics Committee "San Luigi Gonzaga Hospital", protocol number 826/13/08.) in accordance with the International Ethical Guidelines and Declaration of Helsinki.

2.2. Questionnaire

Demographic and health details were collected in the 2009 through an adapted version of the standardized "SIDRIA" questionnaire [34]. The questionnaire was administered to parents, gathering information about demographic, respiratory symptoms and potential risk factors. In particular, parents answered the following specific questions: "has your child ever had diagnosed asthma?"; "did your child have wheezing during the last 12 years?"; "did your child have wheezing after physical activity during the last 12 months?";"did your child have cough at night during the last 12 months?"; "did your child have cough excluding when he had cold, during the last 12 months?"; "did your child usually have phlegm excluding when he had cold, during the last 12 months?"; "did your child usually have sneeze excluding when he had cold?"; "has your child ever had allergic colds?"; "has your child ever had skin redness associated to any other symptoms?"; "has your child ever had bronchitis?"; "has your child ever had asthmatic bronchitis?".

2.3. Spirometry

Spirometry was performed in accordance with ATS/ERS standards [35]. Prior to perform the spirometry test, the instrumental calibration was executed through a 3 L syringe. All measurements were carried out early morning at school, concomitant the scholar activities, at the same time of the survey and of the urine collection. Each child underwent spirometry helped and supervised by a team of pneumologists. The spirometry was performed in standing position wearing a nose-clip and breathing a stead-wills spirometer. Children recorded 3–6 maximum expiratory flow-volume curves in the range of 10–15 minutes and those who had only one acceptable measurement were excluded. Exclusion criteria have been mentioned elsewhere [36]. Forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), forced expiratory flow rate 25–75% ($FEF_{25–75)}$ and maximal expiratory flows were measured at 25%, 50% peaks (FEF_{25} and FEF_{50}, respectively). FEV_1/FVC was obtained as ratio and expressed as percentage.

2.4. Biological Analyses Cotinine and Creatinine

Each subject provided a sample of urine that was aliquoted and stored at −80 °C until biological analysis. Urinary cotinine was measured as biomarker of exoposure to passive and active tobacco smoking, as described elsewhere [37]. Urinary creatinine was quantified by the kinetic Jaffé method and was used to normalise the excretion rate of cotinine, expressed as ng/mg CREA.

2.5. Green Exposure Assessment

The exposure to urban vegetation was assessed using the Normalised Difference Vegetation Index (NDVI) derived from satellite summer images, cloudy-free and referred to the same year of the sampling procedures (2009). NDVI is a commonly used index in epidemiological studies, able to quantify the vegetated biomass, considering that chlorophyll in healthy vegetation mostly reflects the near-infrared band (NIR) (0.7–1.1 μm) compared to the other wavelengths of the light spectrum and, at the same time, strongly absorbs the visible light (0.4–0.7 μm). NDVI is calculated from the ratio of the difference between the NIR and the RED band to their sum and ranges from –1 to 1, where higher positive values indicate vegetation.

In this study, NDVI was derived from Landsat 5 satellite images (resolution 30 m × 30 m) and greenness exposure was calculated for all participants within fixed buffers (300 m radius) around their home address, which has been previously geolocalised.

2.6. Other Variables

The Regional Environmental Protection Agency (ARPA) provided the annual average air pollution concentrations. Hence, the levels of air pollutants, namely PM_{10}, NO_2 and NO, were collected from

five fixed monitoring stations located within the city boundaries, referred to the year of the sampling procedures and expressed as annual averages ($\mu g/m^3$).

2.7. Statistical Analyses

The subjects' characteristics were summarized depending on the variables of interest: as frequencies and percentages, as appropriate for categorical variables, as means (±SD) or medians (±IQR), as appropriate for continuous variables. The Pearson's Chi-squared test, ANOVA or Kruskal Wallis, t-test, or Mann-Whitney tests were used to assess the differences among groups, depending on the type and the distribution of the variables. The associations between greenness exposure and respiratory symptoms were assessed through Odds Ratios (ORs), calculated through logistic regression models using respiratory symptoms (0 = not present; 1 = present) as dependent variables and NDVI, divided into tertiles, as the main independent variable. Logistic regression models were adjusted for age, sex, body mass index (BMI) and urinary cotinine levels. Furthermore, Generalised Linear Models were used to test the association between the respiratory flows measured by spirometry and greenness.

The significance level was set ≤5%. Statistical analyses were performed using IBM SPSS®statistic version 26 integrated with R (3.6.1).

3. Results

Figure 1 depicts children allocations within Turin boundaries, based on the geolocalised home addresses that their parents provided at the survey time. Table 1 summarises the descriptive statistics of the population sample, splitting data between gender categories. Overall, 187 children aged 10–13 years old, 42% females and 58% males were included in the analysis. The sample is homogeneous for age and anthropometric characteristics (BMI, weight and height), exposure to active/passive tobacco smoking measured, as subjectively by questionnaire (passive cigarettes) as objectively, by urinary cotinine. As shown in Table 1, three out of the whole set of respiratory parameters are significantly different between females and males, these latter show a higher mean value of FEV_1, FVC and FEFmax ($p < 0.001$, $p < 0.001$ and $p = 0.010$, respectively). The environmental exposures to average annual air pollutants and to urban vegetation, measured by summertime NDVI, are almost the same between genders.

3.1. Respiratory Symptoms and Greenness

A set of respiratory symptoms has been investigated by collecting details on the respiratory health through the questionnaire. Based on the cross-sectional design of this study, the prevalence of symptoms and respiratory diseases was calculated for current wheezing (21.4), current wheezing occurring after physical activity (5.9), current coughing during the night (20.3), diagnosed asthma ever (9.6), current cough and phlegm not cold-related (9.6 and 9.1, respectively), current sneezing not cold-related (25.7), allergic colds ever (6.4), skin redness ever (17.1), bronchitis and asthmatic bronchitis ever (19.8 and 6.4, respectively) dealing with the research hypothesis: is greenness associated with respiratory health?

NDVI, originally calculated as continuous variable, was divided into tertiles. At this analysis stage, only first and third tertiles were taken into consideration, including 126 children out of 187, who are supposed to represent the lower exposure (1st tertile) and the higher exposure (3rd tertile) to the vegetated areas of the city. The prevalence of respiratory symptoms and asthma between 1st and 3rd tertiles are almost the same compared to those referred to the whole sample: current wheezing (22.4), current wheezing occurring after physical activity (5.6), current coughing during the night (20.0), diagnosed asthma ever (8.9), current cough and phlegm not cold-related (8.8 both), current sneezing not cold-related (23.3), allergic colds ever (6.4), skin redness ever (17.6), bronchitis and asthmatic bronchitis ever (20.8 and 4.8, respectively).

Table 1. Demographics and general characteristic of the whole sample.

Sex n (%)	**Female 79 (42)**	**Male 108 (58)**	***p*-Value**	**All n = 187**
Age (yrs)	11.5 ± 0.7	11.6 ± 0.9	0.465	11.5 ± 0.8
BMI (kg/m^2)	18.6 ± 3.2	19.7 ± 3.8	0.095	19.3 ± 3.6
Weight (kg)	41.6 ± 9.7	45.3 ± 11.9	0.077	43.8 ± 11.2
Height (cm)	148.8 ± 8.5	150.8 ± 9.4	0.205	150 ± 9.1
Passive cigarettes (n/day)	5.1 ± 1.2	4.8 ± 1.3	0.266	4.9 ±1.3
Cotinine (ng/mgCREA)	0.38 ± 1.5	0.36 ± 0.86	0.870	0.61 ± 0.59
FEV_1 (L)	2.2 ± 0.4	2.4 ± 0.5	**<0.001**	2.3 ± 0.5
FVC (L)	2.5 ± 0.5	2.9 ± 0.6	**<0.001**	2.7 ± 0.6
FEV_1/FVC (%)	86.1 ± 7.3	85.7 ± 5.7	0.085	85.9 ± 6.4
FEF_{25} (L/sec)	1.4 ± 0.5	1.6 ± 0.6	0.210	1.5 ± 0.6
$FEF_{25–75}$ (L/sec)	2.5 ± 0.7	2.7 ± 0.8	0.201	2.6 ± 0.7
FEF_{50} (L/sec)	2.9 ± 0.8	3.1 ± 0.8	0.089	3.0 ± 0.8
FEF_{max} (L/sec)	4.3 ± 1.0	4.7 ± 1.0	**0.010**	4.5 ± 1.0
PM_{10} (μg/m^3)	48.7 ± 6.5	48.9 ± 7.0	0.934	48.8 ± 6.8
NO_2 (μg/m^3)	55.8 ± 14.2	55.4 ± 14.8	0.934	55.6 ± 14.5
NO (μg/m^3)	45.3 ± 15.1	45.5 ± 16.2	0.934	45.4 ± 15.7
NDVI	0.25 ± 0.07	0.26 ± 0.07	0.445	0.25 ± 0.07

Table footer: significant *p*-value bolded in the table.

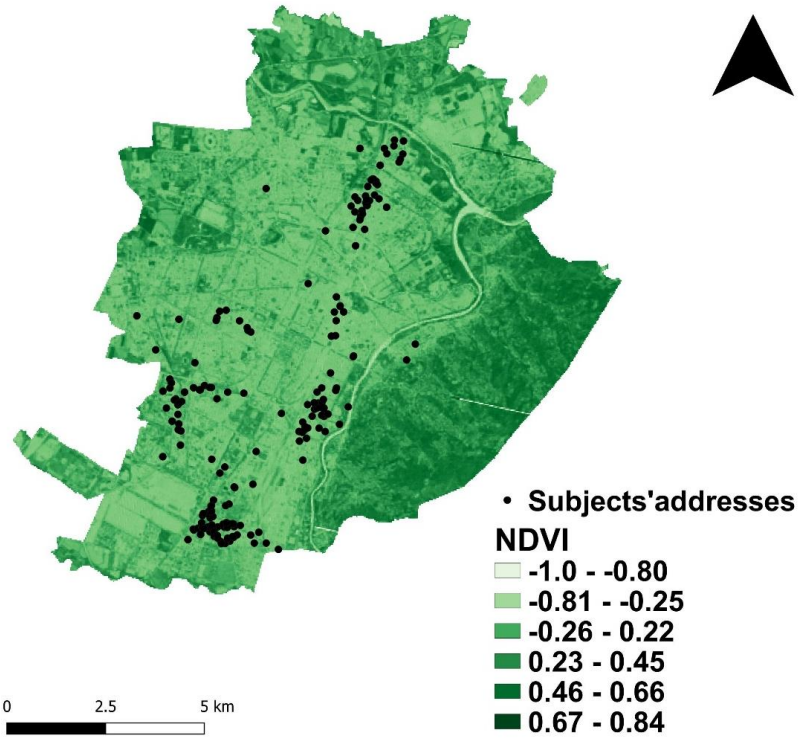

Figure 1. Geolocalised subject's home addresses on the Normalised Difference Vegetation Index (NDVI) maps within Turin boundaries.

Odds Ratios (ORs), summarised in Figure 2, were calculated for both symptoms and respiratory diseases in children lying in 1st and 3rd tertiles. Children included in the 3rd tertile reported significantly fewer ORs for current wheezing [0.25 CI 95% 0.09–0.70, $p = 0.008$], asthma [0.13 CI 95% 0.02–0.7, $p = 0.019$] and bronchitis [0.14 CI 95% 0.05–0.45, $p = 0.001$], compared to children from the 1st tertile

of NDVI. The other respiratory symptoms were not significantly associated to greenness exposure: wheezing after physical activity [0.37 CI 95% 0.06–2.20, $p = 0.271$]; cough at night [0.47 CI 95% 0.18–1.26, $p = 0.271$]; cough [0.70 CI 95% 0.19–2.58, $p = 0.583$]; phlegm [0.79 CI 95% 0.20–3.03, $p = 0.727$]; sneezing [0.88 CI 95% 0.37–2.14, $p = 0.786$], allergic cold [0.61 CI 95% 0.13–2.92, $p = 0.537$]; skin redness [0.54 CI 95% 0.20–1.47, $p = 0.226$] and asthmatic bronchitis [0.17 CI 95% 0.02–1.60, $p = 0.120$].

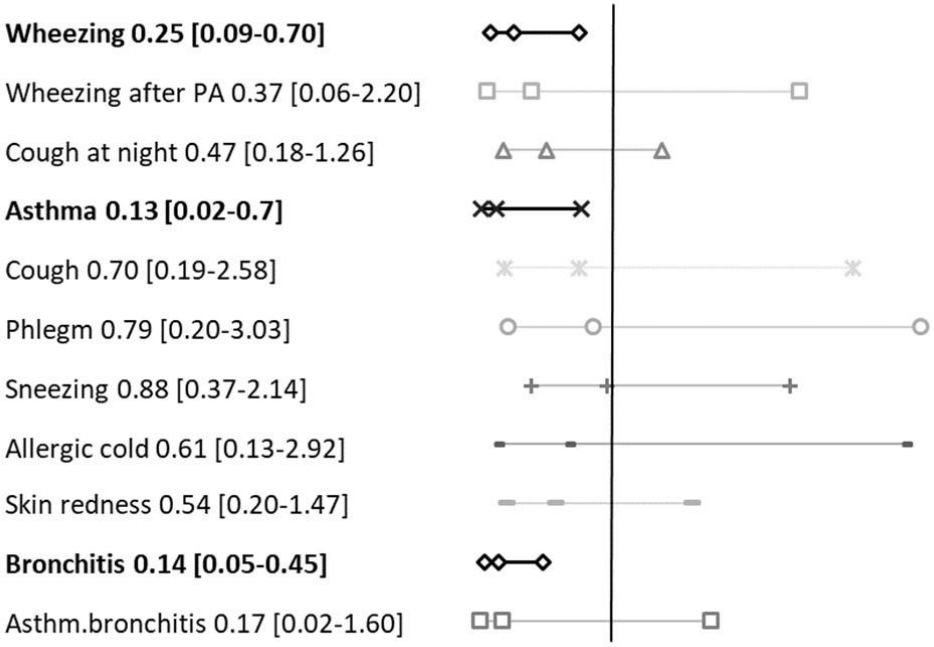

Figure 2. Odds Ratios of symptoms and respiratory disease prevalence referred to children (n = 126) living in more vegetated areas (3rd NDVI tertile) compared to those living in less vegetated areas (1st NDVI tertile).

Similar results have been obtained between crude and adjusted ORs. Controlling for age, sex, BMI and urinary cotinine levels (showed results) strengthened the association and the significance level for both wheezing [unadjusted OR 0.34 CI 95% 0.4–0.86, $p = 0.019$] and bronchitis [unadjusted OR 0.24 CI 95% 0.09–0.63, $p = 0.003$] and slightly modified the relation with asthma [unadjusted OR 0.13 CI 95% 0.01–0.80, $p = 0.009$].

3.2. Respiratory Flows and Greenness

The associations between the respiratory flows and greenness were estimated using several Generalised Linear Models (GLMs), testing each respiratory parameter separated and involving the whole sample of children (n = 187). The Table 2 reports results of the GLM specifically used to estimate the association between greenness (expressed as NDVI divided into tertiles) and $FEF_{25–75}$, set as dependent variable, further controlling for some individual variables (sex, age, BMI) and other environmental conditions, such as tobacco smoking exposure and PM_{10} annual average concentrations. The 3rd tertile of NDVI was set as reference category.

Further results concerning FEF_{25} refer to decreased FEF_{25} in children of the 2nd NDVI tertile compared to those in the 3rd tertile (B: −0.20, C.I.95% −0.40–0.01, $p = 0.039$) and highlight a similar association between greenness and both FEF_{25} and $FEF_{25–75}$.

The analyses on other respiratory parameters did not reach the significance level, but showed a statistical tendency for similar trends of the association between NDVI and lung function. In particular, FEV_1/FVC is diminished in children exposed to lower NDVI values (2nd tertiles) compared to those lying in the 3rd tertile (B = −1.96, C.I.95% −4.22–0.31, $p = 0.091$), controlling for the same set of variables reported in Table 2. Similar results were observed for FEV_1 (B = −0.11, C.I.95% −0.24–0.02, $p = 0.097$).

The exposure to lower NDVI values showed not significant effect on FVC, FEF_{50} and FEFmax (B = −0.07, C.I.95% −0.22–0.9, $p = 0.393$; B = −0.23, C.I.95% −0.51–0.05, $p = 0.105$; B = 0.23, C.I.95% −0.57–0.10, $p = 0.175$, respectively).

Table 2. Generalised Linear Model results, estimating the association between NDVI dividend into tertiles (exposure variable) and forced expiratory flow rate 25–75% ($FEF_{25–75}$) (outcome variable) in the whole sample (n = 187). The 3rd NDVI tertile was set as reference category.

Variables	B	C.I. 95%	*p*-Value
Intercept	2.11	1.32–2.90	**<0.001**
BMI	0.01	−0.02–0.04	0.370
Age	**0.30**	0.18–0.42	**<0.001**
Sex	0.12	−0.08–0.31	0.245
PM_{10}	0.01	−0.01–0.03	0.073
Cigarettes/day	−0.21	−0.01–0.06	0.595
NDVI 1st tertile	0.06	−0.19–0.30	0.640
NDVI 2nd tertile	**−2.40**	−0.48–0.01	**0.049**

Table footer: significant *p*-value bolded in the table.

4. Discussion

In this study, we observed an association among greenness and some respiratory symptoms and diseases, namely current wheezing, asthma and bronchitis, and an association between greenness and $FEF_{25–75}$ and FEF_{25}. In particular, the analysis that involved different levels of exposure to greenness (3rd tertile of NDVI versus 1st tertile) highlighted that children living in greener areas reported less ORs of asthma, current wheezing and bronchitis, even adjusting for age, sex, BMI and cotinine. On the other hand, by analysing the association between greenness and lung function, children living in less vegetated areas (2nd tertile vs 3rd tertile of NDVI) showed a decrease in $FEF_{25–75}$, even controlling for BMI, sex, cigarettes/day and annual average PM_{10} air concentrations.

Our results are in line with some of already published findings. For example, residential surrounding greenness has been previously reported as a protective factor for lifetime wheezing in a population of Mexican children living in Chicago [28]. Tischer et al. [3] observed that children living in the Euro-Siberian area in Spain have reduced risk of wheezing. At this concern, Turin is placed in a different bio-geographic area, namely Continental, which shares only few characteristics with the Euro-Siberian such as the humid climate and the peak in leaves biomass during the summer [38]. Urban areas with greater street tree density were associated with a lower prevalence of asthma in American children, investigated through an ecological approach in New York city [29]. A decreased risk of asthma was also reported in association with an increase in NDVI value at individual level [30]. Moreover, children living in more vegetated areas in New Zeeland showed a lower risk of having asthma [39]. Other authors reported no association between greenness and asthma. A cross-sectional study in Spain found that greenness was not associated with current asthma in children [25] or with asthma prevalence in unadjusted analyses [32].

Concerning the odds for bronchitis in childhood, only one study stated a significantly inverse association of greenness and bronchitis [3]. However, Tischer observed that higher NDVI levels were associated to lower risk of bronchitis only in Spanish children living in the Mediterranean area of the country. The widely held theory that could explain how greenness can improve respiratory health is related to its capacity to enhance environmental biodiversity [40] and reduce exposure to air pollution or even promoting physical activity and prevent overweight and obesity.

On the contrary, our results are in contrast with other studies, which reported that greenness is positively associated with asthma and allergic conditions. Andrusaityte stated that a greater

amount of vegetation is associated with higher relative risk of asthma in Lithuanian children [22]. Living in proximity of the parks was associated with a higher relative prevalence of current asthma in children [25,41]. Many authors suggested few mechanisms by which greenness can act as risk factor in worsening respiratory condition, for example by releasing pollens and fungal spores in the environment [24,42,43], or even increasing the exposure to pesticides and fertilisers [44].

To the best of our knowledge, no already published works investigated the association between greenness and spirometry parameters in children, except for conference abstracts and a study that measured lung functionality through the Forced Oscillation Technique [45].

The fact that NDVI is significantly associated only with $FEF_{25–75}$ and FEF_{25} may indicate a particular susceptibility of small airways under the effect of greenness.

Even if, no statistical significance level was reached, both FEV_1 and FEV_1/FVC are lower in adolescents exposed to lower NDVI values. Therefore, our data suggests that greenness may influence respiratory function as a whole. From this point of view, it would be interesting to undertake future studies using tests that, while analysing the entire respiratory system, allow to specifically focus on the small airways, such as the Forced Oscillation Technique, which seems even more sensitive in detecting the early damage of the airway [46].

5. Strengths and Limitations

The cross-sectional design of the present study is the main limitation that did not allow us to assess the causation direction in general and with clear consequences in the ORs interpretation. Another limitation is that we measured only the exposure to the quantity of green spaces (NDVI) without specifying the type of vegetation and that the sample size might be considered relatively small to investigate all around the lung function.

As remarkable strength, this study is the first that investigated the association between green spaces and respiratory health in this specific geographic area, providing specific data, which respond to the important issue of the geographic variability of these associations [23,27,41,47].

6. Conclusions

Our results support that greenness has an association with respiratory health in children and provide information about a specific geographic area of Italy. Data are in line with some author's results and in contrast with others, indicating that greenness and respiratory health is still poorly understood. Further research is needed to understand the specific mechanism by which urban vegetation may interact with the respiratory health and to enhance the exposure assessment by multi-location assessment and the type characterisation.

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Influence of residential land cover on childhood allergic and respiratory symptoms and diseases: Evidence from 9 European cohorts

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ABSTRACT

Introduction: Recent research focused on the interaction between land cover and the development of allergic and respiratory disease has provided conflicting results and the underlying mechanisms are not fully understood. In particular, green space, which confers an overall positive impact on general health, may be significantly contributing to adverse respiratory health outcomes. This study evaluates associations between surrounding residential land cover (green, grey, agricultural and blue space), including type of forest cover (deciduous, coniferous and mixed), and childhood allergic and respiratory diseases.

Methods: Data from 8063 children, aged 3–14 years, were obtained from nine European population-based studies participating in the HEALS project. Land-cover exposures within a 500 m buffer centred on each child's residential address were computed using data from the Coordination of Information on the Environment (CORINE) program. The associations of allergic and respiratory symptoms (wheeze, asthma, allergic rhinitis and eczema) with land coverage were estimated for each study using logistic regression models, adjusted for sex, age, body mass index, maternal education, parental smoking, and parental history of allergy. Finally, the pooled effects across studies were estimated using meta-analyses.

Results: In the pooled analyses, a 10% increase in green space coverage was significantly associated with a 5.9%–13.0% increase in the odds of wheezing, asthma, and allergic rhinitis, but not eczema. A trend of an inverse relationship between agricultural space and respiratory symptoms was observed, but did not reach statistical significance. In secondary analyses, children living in areas with surrounding coniferous forests had significantly greater odds of reporting wheezing, asthma and allergic rhinitis.

Conclusion: Our results provide further evidence that exposure to green space is associated with increased respiratory disease in children. Additionally, our findings suggest that coniferous forests might be associated with wheezing, asthma and allergic rhinitis. Additional studies evaluating both the type of green space and its use in

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relation to respiratory conditions should be conducted in order to clarify the underlying mechanisms behind associated adverse impacts.

1. Introduction

As urban and suburban environments continue to develop worldwide, understanding the health impacts resulting from different land cover classes is increasingly pertinent. Recent epidemiological studies have evaluated the health impacts of specific land cover types, including urban/grey space, green space, and proximity to agricultural areas or water bodies (blue spaces) (Markevych et al., 2017; Twohig-Bennett and Jones, 2018; van den Berg et al., 2015). Overall, positive health associations have been found with increasing green space exposure, ranging from mental health issues (Engemann et al., 2019) to reductions in diastolic blood pressure, salivary cortisol and heart rate, to decreases in incidence of diabetes, all-cause and cardiovascular mortality (Alcock et al., 2014; Twohig-Bennett and Jones, 2018).

Whether and how green space is related to allergic and respiratory health, however, is unresolved in the literature. While green space may mitigate pollution levels by removing pollutants from the air or by limiting the space available for emission sources, it is also a source of pollens, aggravating allergies and increasing particulate matter counts (van den Bosch and Nieuwenhuijsen, 2017). Forests and soil are a huge source and reservoir of biogenic volatile organic compounds, which can also be detrimental to respiratory health (Gibbs, 2019; Goldstein and Galbally, 2007). Knowledge on how these spaces affect human health is crucial in addressing the increasing worldwide prevalence of asthma and allergies and to direct urban and community planning for prevention (De Marco et al., 2012; Ruokolainen, 2017).

Children are particularly susceptible to the adverse impacts of environmental exposures. At certain early stages of life, when immune and respiratory systems are still developing, exposure to environmental toxins can lead to irreversible health damage (Thurston et al., 2017). Compared to adults, children are generally more active, spend more time outdoors and breathe in more air than adults do in proportion to their weight. Further, allergic and respiratory diseases in paediatric populations have increased in recent decades along with urbanisation (Asher et al., 2006; Pesce et al., 2015).

Studies on the effect of land cover on allergic and respiratory health are increasing, but their results are sometimes contradictory and meta-analyses are often not conclusive. This is presumably due to demographic and/or geographical differences (Fuertes et al., 2014), as well as methodological differences in the assessment of the exposures between cohorts and in the definition of the outcomes across cohorts and studies (Lambert et al., 2017).

The CORINE Land Cover (CLC) is a European wide standardised land cover map spanning a large time scale managed by the European Environmental Agency. It has advantages in that it combines geo-spatial environmental information from national databases and satellite images into 44 land cover classes describing various types of artificial surfaces, agricultural land, forests, wetlands and water bodies (Kosztra et al., 2017).

The aim of this study was to use CLC to estimate the percentage of green, blue, agricultural and grey spaces surrounding the residence of 8063 children, aged 3–14 years, from nine European cohorts to evaluate associations between these four broad land cover types and six indicators of allergy and respiratory health during childhood, including asthma, wheezing, allergic rhinitis and eczema. After individual cohorts were evaluated, meta-analyses were conducted to calculate the pooled effect across studies. As details on the type of vegetation may be crucial in understanding the actual impacts these spaces have on allergy and respiratory outcomes (Gernes et al., 2019), a secondary analysis was also conducted to further investigate the effects of different types of tree cover on health outcomes by taking advantage of the distinction CLC provides between coniferous and deciduous forests.

2. Materials and methods

2.1. Study population

The present study considers nine different cohorts across four European countries (Italy, France, Slovenia and Poland) that have contributed to the FP7 HEALS (Health and Environment-wide Associations based on Large population Surveys) project, aimed at assessing health-environment associations through an exposome approach (Wild, 2012). Detailed descriptions of the nine cohorts are reported in the Online Supplement (Text S1). From these, a subsample of 8063 children with an age range of 3–14 years was selected based on the availability of information on wheezing, asthma and respiratory/allergic diseases and geocoded residential addresses:

1) 877 children (3–8 yrs) enrolled at birth in 2003–2006 and living in Nancy (Northeastern France) and Poitiers (Central France) from the EDEN study (Etude des Déterminantspré et post natals du développement et de la santé de l'Enfant) (Heude et al., 2016);
2) 748 schoolchildren (3–14 yrs) enrolled in 2009–2010 and living in the Province of Verona (Veneto, Northern Italy) from the Fumane & Mezzane di Sotto cross-sectional study (Marcon et al., 2014);
3) 1627 twins (age 3–14 yrs) enrolled in the Italian Twin Registry (ITR) study beginning in 2001 and living throughout Italy (Brescianini et al., 2013);
4) 274 twins (mean age 3 yrs) enrolled at birth in 2009 and living throughout Italy from the MUltipleBIrthCOhort (MUBICOS) study (Brescianini et al., 2013). MUBICOS is part of the ITR but these cohorts do not overlap;
5) 167 children (age 7–8 years) recruited at birth in 2008–2009 from Ljubljana and its surroundings from the Slovenian study as part of the PHIME study (PHIME-SLO) (Miklavčič et al., 2011; Valent et al., 2013);
6) 135 children (8–14 yrs) enrolled in 1991–1993 and living in Pisa (Tuscany, Central Italy) from the Pisa-2 cross-sectional study (Maio et al., 2016; Nuvolone et al., 2011);
7) 78 children (7 yrs) enrolled during the first trimester of pregnancy in 2007 and living in Lodz district, Poland from the Polish Mother and Child Cohort (REPRO_PL) study (Polańska et al., 2016, 2011);
8) 393 schoolchildren (10–13 yrs) enrolled in 2003, followed-up in 2010 and living in Turin (Piedmont, Northern Italy) from the Turin cohort study (Piccioni et al., 2015);
9) 3764 schoolchildren (3–14 yrs) enrolled in 2006 and living in the district of Viadana (Mantua (Lombardy, Northern Italy) from the Viadana cross-sectional study (de Marco et al., 2010).

In each of the original studies, ethical approval was obtained from the local ethics committees. Fig. 1 shows the geographical distribution of the children included, differentiated by study.

2.2. Health outcomes and covariates

In all studies, data on health outcomes, as well as on potential confounders, were collected through parental questionnaires.

Children were classified as having:

- "*lifetime wheeze*" (wheeze) if children were reported to have ever

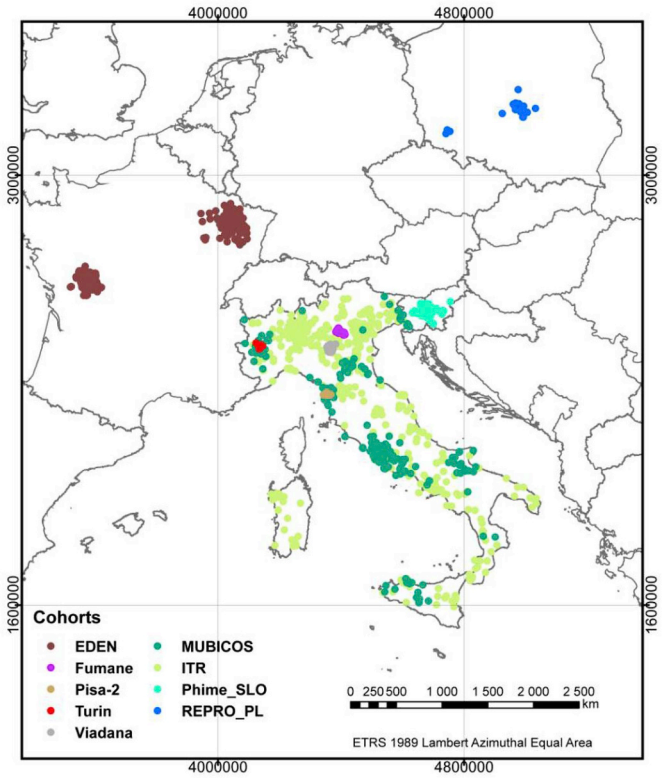

Fig. 1. Geographical distribution of the children included in the analyses differentiated by study.

had wheezing or whistling in the chest at any time in the past;
- "*current wheeze*" if the child had any wheezing in the last 12 months;
- "*lifetime asthma*" (asthma) if the child had asthma at any time in the past;
- "*current asthma*" if the child was currently taking medication for asthma and/or suffered an asthma attack in the last 12 months;
- "*lifetime allergic rhinitis*" if the child ever had any nasal allergy or hay fever at any time of the past;
- "*eczema*" if the child ever had an itchy rash on one or more parts of the skin which was coming and going or had been diagnosed with eczema.

The variables used in the assessment of the outcomes differed slightly across the studies. Details on the specific questions used in each study are described in the Online Supplement (Table S1). Child's age (years), sex, body mass index (BMI), parental history of allergy or asthma, parental smoking as a proxy of passive smoke exposure, maternal education ("high" if the mother has a high school diploma or higher qualification vs. "low"), used as proxy of socio-economic status, were considered as potential confounders in the models used to determine the associations between land covers and health outcomes.

2.3. Residential land cover

The participant's residential addresses were geocoded and re-projected into CLC's Lambert equal area projection (Kosztra et al., 2017). The proportion of each CLC class surrounding each residential location was calculated by using buffer zones with four radii: 100 m, 300 m, 500 m, and 1000 m. The raster version of CLC with a pixel size of 100 m was used. Depending on the year of data collection for the specific cohort study, the nearest CLC layer was selected from the available 1990, 2000, 2006, 2012 or 2018 years: 1990 for Pisa-2, 2006 for Eden, Fumane, PHIME-SLO, Turin and Viadana, and 2012 for Mubicos and REPRO_PL. 2012 was selected for ITR as it was the year closest to the visit dates of the children selected for this study.

Table 1
Description of land cover features according to CORINE classification.

Land cover feature	CORINE code	Description
Green space	1.4.1	Green urban areas
	1.4.2	Sport and leisure facilities
	3.1.1	Broad-leaved forest
	3.1.2	Coniferous forest
	3.1.3	Mixed forest
	3.2.1	Natural grassland
	3.2.2	Moors and heathland
	3.2.3	Sclerophyllous vegetation
	3.2.4	Transitional woodland/shrub
Grey space	1.1.1	Continuous urban fabric
	1.1.2	Discontinuous urban fabric
	1.2.1	Industrial or commercial units
	1.2.2	Road and rail networks and associated land
	1.2.3	Port areas
	1.2.4	Airports
	1.3.1	Mineral extraction sites
	1.3.2	Dump sites
	1.3.3	Construction sites
Blue space	5.1.1	Water courses
	5.1.2	Water bodies
	5.2.1	Coastal lagoons
	5.2.2	Estuaries
	5.2.3	Sea and ocean
Agricultural space	2.1.1	Non-irrigated arable land
	2.1.2	Permanently irrigated land
	2.1.3	Rice fields
	2.2.1	Vineyards
	2.2.2	Fruit trees and berry plantations
	2.2.3	Olive groves
	2.3.1	Pastures
	2.4.2	Complex cultivation patterns
	2.4.1	Annual crops associated with permanent crops
	2.4.3	Land occupied by agriculture, with areas of natural vegetation
	2.4.4	Agro-forestry areas
Forest	3.1.1	Broad-leaf forest
	3.1.2	Coniferous forest
	3.1.3	Mixed Forest
Broad-leaf forest	3.1.1	Broad-leaf forest
Coniferous forest	3.1.2	Coniferous forest
Mixed Forest	3.1.3	Mixed Forest

From the resulting proportions of original CLC classes, eight land cover features used in this study were calculated: percentages of green space, blue space, grey space, agricultural space, forest cover, coniferous forests, deciduous forests and mixed forests. Note that green spaces do not contain agricultural spaces. Table 1 shows which original CLC classes make up each of the calculated exposure variables.

2.4. Statistical analysis

To minimise bias from the heterogeneity of methodological protocols among studies, we used a two-stage approach for analysing individual participant data and calculating the pooled effect across studies (Fisher, 2015; Fuertes et al., 2016). In the first stage, associations of health outcomes with each of the land coverage indicators were estimated within each study using logistic regression models, adjusting for the potential confounders available in each study (sex, age, BMI, parental history of allergy, maternal education, parental smoking), and were expressed using odds ratios (OR) with corresponding 95% confidence intervals (95% CI). Cluster-robust standard errors were included in twin studies (i.e. ITR and Mubicos) to take into account any

correlations between twin siblings. Land-cover main classes (i.e. green, grey, blue, and agricultural) were included in the model as continuous variables, and OR were estimated for a 10% increase of land coverage within a 500 m buffer. In each model, associations with health outcomes were estimated only in studies that had more than 10 cases in order to avoid data sparseness.

In the second stage, fixed-effects meta-analyses were performed on the estimates calculated for individual studies using the inverse-variance method and overall OR were calculated. Fixed-effects meta-analyses were adopted under the assumption that land cover features have the same effects on health outcomes across all studies. Random effects meta-analyses using the DerSimonian and Laird methods were performed when a significant heterogeneity across the studies emerged (Fisher, 2015).

The associations of health outcomes with a binary indicator of presence/absence of forests (any, coniferous, broad-leaf, mixed) within a 500m buffer surrounding the children's home were also evaluated in secondary analyses.

As diagnosis of asthma can be difficult in very young children (Bacharier et al., 2008), analyses on current and lifetime asthma were restricted to children aged 6 and older.

A 0.05 significance level was adopted. All statistical analyses were performed with STATA 14.2.

2.5. Sensitivity analyses

As health outcomes may have different clinical phenotypes that vary by age, we performed sensitivity analyses in which the children were stratified into three homogeneous age-groups (3–5; 6–10; 11–14 years), to further investigate whether land cover features were differently associated with the outcomes across these groups. To evaluate the potential interaction between sex and green space exposure, the models were also run separately for males and females. Additionally, to test the stability of the associations found in the main analyses, we performed two further sensitivity analyses using different indicators of exposure: 1) using a binary indicator (i.e. 1 = high vs. 0 = low exposure) based on intra-study median coverage for each type of land-cover class; 2) adopting different distance buffers (100, 300 and 1000 m) for land-coverage around the home address. To check whether the inclusion of both twins in a pair might have over-weighted the Mubicos and ITR studies, we also performed a further sensitivity analysis where one child per pair was randomly excluded from the models.

Finally, the analyses of association of green, grey and agricultural space with respiratory outcomes were further adjusted for estimates of outdoor exposure to NO_2 (annual average concentrations) at the home addresses. These analyses were conducted for the EDEN and Viadana cohorts only (Heude et al., 2016; Marcon et al., 2014), since data on air pollution were not available for the other studies. For EDEN, a sensitivity analysis adjusted for PM_{10} exposure was also conducted. The methods for the exposure assessment and attribution are described in the Online Supplement (Text S2).

3. Results

Overall, 8063 children were geo-referenced and included in the analyses (Fig. 1). The Viadana study contained the highest number of children (n = 3764). Females represented 47.7% of the overall sample, and the median age ranged from 3 years in the Mubicos study to 12 years in the Turin study. The children from the Pisa-2 study, which was the oldest study included (1991–1993), were the most exposed to passive smoking, but also those with lowest parental history of allergy and lowest parental education (Table 2).

The distribution of respiratory and allergic health outcomes was significantly heterogeneous across studies. Children from the EDEN study in France, had the greatest prevalence for all six of the considered outcomes, with a prevalence of asthma and allergic rhinitis of 15.5% and 20.8%, respectively, and a prevalence of wheezing and eczema above 40%.

The distribution of land coverage around children's homes by buffer radius and study is shown in Table 3. In all studies, as the radius of the buffer around the children's home increased, the proportion of land covered by urban grey space decreased and, consequently, the proportion of green, blue and agricultural spaces increased. Within a 500 m radius buffer, the children recruited in the *Turin study* were the most exposed to urban grey space (land coverage: 94.4%), while the children from *Fumane* were the most exposed to green and agricultural space (12.6% and 59.6%, respectively). The children from the *Pisa-2 study* had no detectable green space within 500 m from their home, while those from *Turin* had an average proportion of agricultural space below 1%. In all studies, the proportion of blue space was extremely low, ranging from 0 to 1.1%. The correlation matrices showing the pairwise relation between different types of land cover and between different

Table 2
Characteristics of the children included in the present study. Data expressed as n (%) or median [range]. †children aged 6 and older. *Studies with < 10 cases for one of the outcomes were excluded from the meta-analyses to avoid data sparseness.

Survey	EDEN	Fumane	ITR	Mubicos	Phime-SLO	Pisa-2	REPRO_PL	Turin	Viadana
n	877	748	1627	274	167	135	78	393	3766
Country	France	Italy	Italy	Italy	Slovenia	Italy	Poland	Italy	Italy
Data collection	2003–2014	2010	2001-ongoing	2009–2014	2007-ongoing	1991–1993	2014–2019	2010	2006
Subject characteristics									
Age (years)	8 [3–8]	9 [3–14]	10 [3–14]	3 [-]	8 [7–8]	12 [8–14]	7 [-]	12 [10–13]	9 [3–14]
Female sex	445 (50.7%)	365 (48.8%)	805 (49.5%)	127 (46.4%)	84 (50.3%)	55 (40.7%)	44 (56.4%)	174 (44.3%)	1745 (46.3%)
BMI (kg/m2)	15.6 [10.2–21.6]	17.0 [8.9–27.7]	17.4 [9.0–26.1]	11.3 [8.4–16.2]	15.8 [12.4–22.4]	19.1 [14.6–30.2]	15.9 [13.2–24.1]	18.3 [12.2–28.6]	17.2 [7.8–27.9]
Parental history of allergy	310 (35.4%)	254 (34.0%)	496 (30.5%)	42 (15.3%)	–	19 (14.1%)	12 (15.4%)	132 (33.6%)	846 (22.5%)
Passive smoking exposure	297 (33.9%)	351 (46.9%)	680 (41.8%)	38 (13.9%)	12 (7.2%)	75 (55.6%)	20 (25.6%)	94 (23.9%)	1930 (51.3%)
Maternal education (high)	725 (82.7%)	484 (64.7%)	–	264 (96.4%)	119 (71.3%)	48 (35.6%)	72 (92.3%)	280 (71.2%)	1941 (51.6%)
Outcomes									
Wheezing	362 (41.6%)	179 (24.5%)	494 (31.2%)	93 (33.9%)	–	20 (14.8%)	–	95 (24.7%)	874 (23.8%)
Current wheezing	100 (11.5%)	56 (7.6%)	135 (8.6%)	51 (18.6%)	–	–	–	23 (5.9%)	280 (7.6%)
Asthma†	110 (15.5%)	63 (9.8%)	129 (9.6%)	–	*7 (4.2%)**	14 (10.4%)	*8 (10.3%)**	46 (11.8%)	322 (6.9%)
Current asthma†	57 (8.0%)	43 (6.7%)	54 (4.0%)	–	–	*8 (5.9%)**	–	22 (6.0%)	136 (4.4%)
Allergic rhinitis	182 (20.8%)	78 (10.5%)	222 (13.9%)	–	–	19 (14.1%)	–	35 (9.0%)	303 (8.2%)
Eczema	361 (41.2%)	164 (22.0%)	238 (16.4%)	40 (17.8%)	37 (22.2%)	10 (7.4%)	21 (26.9%)	69 (17.6%)	801 (21.7%)

Land coverage around children's home.

Table 3
Land coverage around children's home by buffer radius and study. Data expressed as a percentage of land coverage, mean ± SD. Bold represents the chosen buffer for the primary analysis.

Survey	EDEN	Fumane	ITR	Mubicos	Phime	Pisa-2	REPRO-PL	Turin	Viadana
n	877	748	1627	274	167	135	78	393	3766
Grey space									
< 100 m	74.7 ± 37.4	46.0 ± 42.6	89.0 ± 26.0	72.4 ± 40.8	66.4 ± 43.6	85.2 ± 29.2	93.2 ± 19.8	97.8 ± 11.7	68.6 ± 37.9
< 300 m	67.6 ± 32.4	36.7 ± 31.6	81.6 ± 26.5	67.8 ± 36.8	61.7 ± 38.6	76.5 ± 24.9	87.4 ± 21.6	96.3 ± 11.6	56.7 ± 30.0
< 500 m	**60.0 ± 31.8**	**27.7 ± 22.7**	**76.4 ± 26.5**	**62.5 ± 34.7**	**55.5 ± 36.3**	**66.9 ± 22.1**	**83.1 ± 22.5**	**94.4 ± 12.3**	**45.7 ± 25.3**
< 1 km	47.2 ± 32.2	14.2 ± 11.9	67.0 ± 26.9	52.0 ± 32.3	44.1 ± 33.7	52.6 ± 20.3	76.1 ± 21.3	90.3 ± 12.0	41.7 ± 30.4
Green space									
< 100 m	2.5 ± 12.4	6.2 ± 18.0	1.7 ± 10.1	5.5 ± 21.5	6.6 ± 20.7	0	1.7 ± 7.6	1.4 ± 8.4	1.0 ± 6.2
< 300 m	4.1 ± 11.2	9.2 ± 15.7	2.9 ± 10.2	5.6 ± 17.5	9.1 ± 19.0	0	4.7 ± 8.9	2.5 ± 8.0	2.6 ± 8.2
< 500 m	**6.1 ± 11.8**	**12.6 ± 16.4**	**4.2 ± 10.7**	**6.7 ± 16.7**	**12.4 ± 19.6**	**0**	**8.1 ± 11.0**	**4.0 ± 8.8**	**4.5 ± 10.0**
< 1 km	10.1 ± 13.0	22.3 ± 13.0	6.7 ± 12.4	8.6 ± 16.6	21.2 ± 21.8	0.0 ± 0.1	12.9 ± 12.7	6.3 ± 7.5	7.7 ± 11.7
Blue space									
< 100 m	0.5 ± 5.1	0	0.1 ± 1.5	0	0.2 ± 1.8	0.6 ± 3.8	0	0.4 ± 5.0	0.1 ± 2.2
< 300 m	0.6 ± 4.2	0	0.4 ± 3.2	0.5 ± 3.6	0.4 ± 2.5	1.3 ± 4.4	0	0.7 ± 4.1	0.1 ± 1.0
< 500 m	**0.7 ± 3.8**	**0.0 ± 0.3**	**0.7 ± 4.0**	**0.9 ± 5.0**	**0.7 ± 2.6**	**1.1 ± 3.0**	**0**	**1.0 ± 3.9**	**0.3 ± 2.1**
< 1 km	1.0 ± 3.5	0.0 ± 0.3	1.5 ± 5.5	1.8 ± 6.8	0.8 ± 2.1	1.8 ± 2.1	0	1.4 ± 3.3	1.2 ± 3.5
Agricultural space									
< 100 m	22.3 ± 35.8	47.8 ± 41.4	9.0 ± 23.6	22.1 ± 37.2	26.8 ± 39.5	14.2 ± 28.9	5.0 ± 18.8	0.4 ± 5.0	30.3 ± 37.8
< 300 m	27.6 ± 31.2	54.1 ± 29.2	14.8 ± 24.2	26.0 ± 33.6	28.8 ± 32.6	22.2 ± 24.7	7.9 ± 20.2	0.5 ± 4.7	40.5 ± 30.7
< 500 m	**33.2 ± 31.0**	**59.6 ± 18.5**	**18.5 ± 24.2**	**29.7 ± 32.6**	**31.4 ± 28.5**	**32.1 ± 22.0**	**8.8 ± 20.3**	**0.7 ± 4.9**	**49.5 ± 27.9**
< 1 km	41.6 ± 31.8	63.5 ± 12.2	24.6 ± 25.0	37.2 ± 31.4	33.8 ± 24.4	45.6 ± 20.6	11.0 ± 18.2	1.9 ± 5.7	63.2 ± 25.0

Associations of green, grey, blue and agricultural space exposure with allergic and respiratory health outcomes.

buffer sizes are shown, respectively, in Fig. S1 and Fig. S2 in the Online Supplement.

In the primary analyses, we investigated the associations between residential surrounding land cover (e.g. green, grey, blue and agriculture spaces) within a 500 m radius buffer and six allergic and respiratory health outcomes. These analyses were done across participant studies, for a total of 24 meta-analyses (all forest plots are shown in the Online Supplement, Figs. S3–S6). Overall, a median of 6066 children (range: 4814 to 6806) and 5.5 studies (range: 4 to 9) were included in the meta-analyses, depending on the outcome and land cover variables. The meta-analysis results are summarised in Table S2 in the Online Supplement.

The proportion of land covered by green space was significantly associated with increased odds of lifetime and current wheezing (+5.9% and +13.0%, respectively, per 10% increase in green-covered space), as well as with lifetime and current asthma and allergic rhinitis (+9.2%, +12.1%, and +8.1 respectively) (Fig. 2). No significant associations were found between land cover classes and eczema. As significant heterogeneity between studies was found in the association between green space and lifetime wheezing, the analysis was repeated using random-effects meta-analysis. The results were consistent with the main analysis (OR = 1.06, 95% CI:0.98–1.15).

The associations between green space and health outcomes were similar in males and females and across different ages groups (Fig. S7 and Table S3 in the Online Supplement), indicating that there was no interaction between sex, age and exposure to green space on the considered outcomes. No statistically significant associations were found between exposures to urban grey and blue spaces in relation with any of the health outcomes tested in the meta-analyses. Agricultural space had a borderline negative association with lifetime wheezing, and was

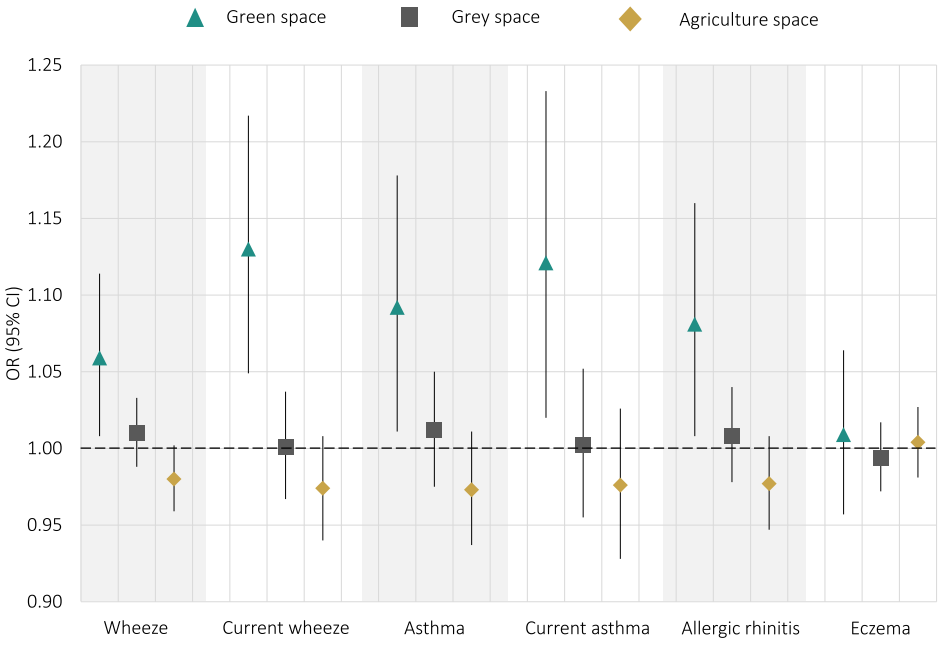

Fig. 2. Associations between land coverage within 500 m from children's home and allergic and respiratory outcomes. Odds ratios (OR with 95% confidence interval, CI) are estimated for a 10% increase of land covered by green, grey, or agricultural space. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

overall moderately protective, although not at statistically significant level, in all the considered respiratory outcomes.

The associations between green space exposure and respiratory outcomes were overall confirmed in all the sensitivity analyses: 1) When using a binary indicator for exposure based on intra-study median exposure (high vs. low exposure, Table S4 in the Online Supplement); 2) when using different buffer radii (100 m, 300 m, and 1000 m) (Table S5); and 3) when one of the twins from the twin cohorts (ITR and Mubicos) was randomly excluded from the models (Table S6).

Further adjustment of the analyses for residential outdoor NO_2 levels (in Viadana and EDEN, Fig. S8) or PM_{10} (in EDEN only, data not shown) did not change the estimates for the associations between land cover features and health outcomes.

The presence of forests was investigated within 500 m from children's residential address and is presented in (Table 4). The children sampled in the Fumane study were the most exposed to forests (any type), while children from Pisa-2 were not exposed to forests within 500 m of their home. Children sampled in the Viadana and in the Turin studies, moreover, did not have any coniferous or mixed forests near home.

After adjusting meta-analyses for potential confounders, children with forests near their homes had a 26% greater odds of having current wheeze compared to those who lived further (OR = 1.26; 95% CI: 1.01–1.56; p = 0.039) (Fig. 3 and Table S7). After separating between type of tree cover, children living close to a coniferous forest had greater odds of having current wheeze (OR = 1.76; 95% CI:1.05–2.97), lifetime wheeze (OR = 3.95; 95% CI: 2.08–7.49), current asthma (OR = 4.45; 95%CI: 1.81–10.9), lifetime asthma (OR = 2.54; 95% CI: 1.10–5.82) and allergic rhinitis (OR = 3.39; 95% CI: 1.83–6.30) than those living further (all p < 0.05). The detailed forest plots for the meta-analyses are displayed in Figs. S9–S12 in the Online Supplement.

4. Discussion

Our study set out to investigate whether residential land cover was associated with the occurrence of respiratory and allergic symptoms in children. Meta-analyses were conducted based on data collected from nine paediatric cohorts from four European countries. We found that greater residential exposure to green space was associated with increased odds of wheezing and asthma (both current and lifetime) and lifetime allergic rhinitis, but not with eczema. Children living within a 500 m buffer including coniferous forests, in particular, were found to have odds of wheeze, asthma and allergic rhinitis that were 2- to 4-fold higher than those who did not. Grey (urban) and blue space were not associated with any of the studied outcomes, while agricultural space was moderately protective, although not at a statistically significant level, for all the respiratory outcomes.

Many studies investigating the impacts of green space have shown a largely beneficial influence on various health outcomes including wellbeing, diastolic blood pressure, salivary cortisol, heart rate, incidence of diabetes, all cause and cardiovascular mortality (Cilluffo et al., 2018; Engemann et al., 2019; Twohig-Bennett and Jones, 2018; van den Berg et al., 2015). Our results suggest that, contrary to impacts on these other health indicators, allergic and respiratory symptoms can increase with increased nearby green space and are consistent with many, but not all, studies focused on similar associations (Gernes et al., 2019; Lambert et al., 2017; Tischer et al., 2017).

Two questions arise: First, what underlying mechanisms cause green space to confer a negative impact to respiratory and allergic outcomes in children, and second, what is the reason for inconsistent results between studies?

Green spaces may be problematic for respiratory health because they are sources of VOC emissions, pollen, moulds, and aerosols, all of which have been shown to create allergies and respiratory health problems (Annesi-Maesano, 2013; Cecchi et al., 2018; Gibbs, 2019; Marchetti et al., 2017; Schuler IV and Montejo, 2019). There are also ways green space can be beneficial to respiratory health, by providing some protection from anthropogenic air pollution through absorption, providing physical barriers against emission sources, or by limiting the overall area available to sources of pollution such as traffic or buildings (van den Bosch and Nieuwenhuijsen, 2017).

Our results showing increased odds for asthma and allergic rhinitis confirm previous results by some authors (Andrusaityte et al., 2016; Dadvand et al., 2015; Tischer et al., 2017). However, they are also contradictory to results from others (Alcock et al., 2017; Gernes et al., 2019; Lovasi et al., 2008; Sbihi et al., 2015; Tischer et al., 2018). When considering other meta-analyses, that conducted by (Lambert et al., 2017) found too many inconsistencies between studies to accurately assess possible associations and that done by (Fuertes et al., 2016) found differential associations with NDVI within a 500 m buffer and allergic rhinitis depending on the cohort and no significant associations from meta-analyses.

These differences between the results showing positive or negative associations between allergic and respiratory symptoms and green space could be partially due to differences in cohorts or age groups, and inclusion of confounding variables. More likely, in our view, the inconsistencies in exposure definition may be driving this difference. The term “green space” itself may be part of the problem, as it can refer to multiple types of land cover, from homogenous grass fields to highly diverse forests. Taylor and Hochuli find that the term “green space” used in the literature can have up to six different meanings, largely falling into two categories describing either naturally vegetated areas or urban green space (Taylor and Hochuli, 2017). These two categories are both considered “green space”, however they may impart different impacts. Of the similar studies looking into respiratory health, most use NDVI as an indicator of green space which covers both natural vegetation and urban green space. While NDVI is a specific measure with a clear definition, the values change throughout the year depending on vegetation growth and seasonal effects. In wide area studies covering different years and seasons it is difficult to have consistent calibrated NDVI values, even if annual averages are used. In the present study, the decision to include exposures based on CLC rather than NDVI was made based on the capacity of CLC to separate between, and even within, agricultural, forest and grass areas, however this makes direct comparison between many other studies difficult.

In considering the distinction between forest types, we found significant associations between coniferous forests and respiratory health outcomes, although the number of children living in proximity to these forest types was relatively small in our study. To our knowledge, our study is the first to suggest an adverse significant relationship between living close to coniferous forests and respiratory health. It is not clear, however, if this result is due to something specific to conifers

Table 4
Forest-covered land near children's residential home by study. Data indicate the number (%) of children who live within 500 m from a forest.

Survey	EDEN	Fumane	ITR	Mubicos	Phime	Pisa-2	REPRO-PL	Turin	Viadana
n	877	748	1627	318	167	135	78	393	3766
Forest (any)	270 (30.8)	483 (64.6)	178 (10.9)	50 (16.4)	89 (53.3)	0	6 (7.7)	12 (3.1)	1048 (27.9)
Broad-leaf forest	250 (28.5)	400 (53.5)	144 (8.9)	36 (13.1)	33 (19.8)	0	1 (1.3)	12 (3.1)	1048 (27.9)
Coniferous forest	14 (1.6)	32 (4.3)	22 (1.4)	6 (2.2)	19 (11.4)	0	2 (2.6)	0	0
Mixed forest	12 (1.4)	141 (18.9)	30 (1.8)	26 (9.5)	74 (44.3)	0	4 (5.1)	0	0

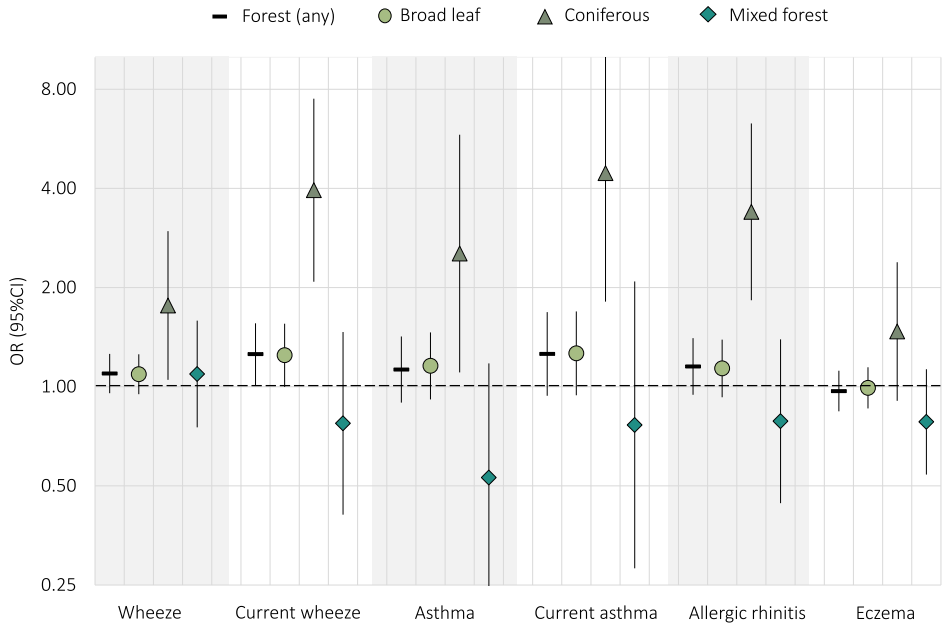

Fig. 3. Associations between proximity to forests and respiratory and allergic symptoms. Odds ratios (OR with 95% confidence interval, CI) indicate the risk for children who live within 500 m from a forest vs. those who live further.

themselves, or potential differences in pollens, humidity, or mould spores (Kurlandsky et al., 2011). We note that most of our study participants were from Italy, and that no cohorts were examined from Nordic countries where coniferous forests are dominant. Sensitization to conifer pollens – especially from the cypress and pine families - among allergic patients, however, is highly prevalent also in Mediterranean areas (Domínguez-Ortega et al., 2017; Gastaminza et al., 2009; Marchetti et al., 2017) and the prevalence of sensitization to these types of pollens have been increasing in the last decades (Charpin et al., 2005).

No associations were found between grey or blue space for any of the allergic or respiratory outcomes. In terms of blue space, a conclusive statement cannot be made because of the lack statistical power. For grey spaces, our results are consistent with pooled analyses of four studies on Spanish children done by (Tischer et al., 2017), who found no statistically significant associations for grey space determined by CLC within 300 m buffers and wheezing, asthma and allergic rhinitis. Another study by (Ebisu et al., 2011) found a significant association between urban land use and wheeze severity in American infants, but the significance of this association disappeared when the models were adjusted for NO_2.

Again, here is the question regarding the definition of exposure. In the case of (Tischer et al., 2017) where CLC was also used at a buffer radius of 300 m, our results are also consistent with no association between grey space and any of the allergic and respiratory outcomes. In the case of (Ebisu et al., 2011), their exposures are estimated from the U.S. National Land Cover Database, which may differ from CLC, and they also considered a much larger buffer surrounding the residence (1540 m). Further, it may be problematic to compare urban areas in Europe to those in the United States as the urban topography and green spaces are dissimilar.

Several studies have explored the benefits of rural and agricultural exposures on the immune system and respiratory health due to the influence of the local microbiome and its impact on immunoregulation (Deckers et al., 2019; Frei et al., 2019; Hanski et al., 2012). When evaluating agricultural space, our results show a moderately protective effect for all the respiratory outcomes, although not at a statistically significant level.

4.1. Strengths and limitations

Our analyses used CLC to define exposures to green space and other land cover features. As our cohorts span across four countries, the standardization of the exposures across Europe provided by CLC allows for pooled results and consistency across studies.

Another significant advantage of CLC is that it can distinguish green spaces between coniferous, deciduous and mixed forests, a necessary step towards understanding the health effects of specific vegetation types and untangling the complexities inherent in the interactions between respiratory health and green space. To our knowledge, our study presents the first results to assess the associations between the type of nearby forest cover and allergic and respiratory impacts. It is also, to our knowledge, the first study to use CLC to estimate residential green space in relation to health outcomes. The use of CLC, rather than NDVI to determine green space exposure, however, means we cannot directly compare our results with many other studies. CLC data does not capture small green spaces well which some have suggested renders its use inappropriate in urban areas (Annerstedt et al., 2012; Mitchell et al., 2011). Consequently, our observations may be confounded due to exposure misclassification.

Quantifying land cover is only an indirect way of assessing exposure. The chosen buffer size can also affect the results. Our choice of a 500 m buffer is supported by (Browning and Lee, 2017), and our results were consistent when changing the buffer radius to 300 m or 1000 m (Table S5 in the Online Supplement). A further concern is that CLC is only available for specific years, which may mean that for some of the younger children the exposure was measured before they were born. In most cases, however, the CLC values for most areas from 2006 to 2012, for example, do not change enough to suggest this is an issue, and we can assume CLC values are relatively constant over our time period for most areas.

This study meta-analyses heterogeneous population-based paediatric cohorts with different protocols, outcome definitions and with different age-ranges. The differences in age across cohorts could be of concern, as the relation between the clinical phenotypes and the exposure might vary by age. However, we found that the associations between type of land cover and health outcomes were homogeneous across three different age groups (3–5, 6–10, and 11–14 years; Table S3

in the Online Supplement) suggesting that CLC exposures have similar effects on respiratory symptoms within the age-range of 3–14 years. The definitions adopted for asthma, wheezing, allergic rhinitis, and eczema in all studies were validated in previous studies. The differences in the wording across the cohorts are minor and, moreover, the meta-analyses showed an overall great homogeneity across studies in the associations between land cover features and outcomes, suggesting that differences in protocols and definitions might have biased our estimates only to a minor extent.

Two considerable limitations of our study are that daily activity of participants has not been assessed and that only current residential exposure is explored. We acknowledge that moving behaviour has not been assessed. Differences between cases and non-cases (e.g. if families of children with wheezing and rhinitis were more likely to move to greener areas) could affect the estimates of our study or provoke a reverse causation. However, we observed that the effect size of the associations of green-space with respiratory and allergic symptoms was stronger when looking at the outcomes reported in the last 12 months, during which the likelihood of having moved is lower as compared to the lifetime outcomes. This suggests that there has not been a differential error according to the disease status of the children and, as a consequence, it is more likely that the lack of information on house moving history has biased our results toward the null effect by reducing the strength of our associations. Moreover, we showed that the associations were homogeneous across different age groups (Table S3 in the Online Supplement). As these age groups should be homogeneous in terms of the risk of moving (e.g. children within the group 11–14 years all have a similar probability to have moved in their lifetime, and have greater probability than children within the group 3–5 years), the homogeneity across these three groups indicates that, if moving behaviour has influenced our results, it has biased them only to a minor extent.

The number of children that were exposed to some forest types, especially coniferous and mixed forests, was relatively small, affecting the statistical power of the analyses. This is likely to increase the risk of type-2 error, e.g. not being able to find an association where there is actually one ("false-negative conclusion"), and may have prevented potentially significant associations. Moreover, as low power is affecting the size of the confidence intervals of the associations, it should be taken into account when interpreting our results, and both significant and non-significant results should be interpreted with caution.

Finally, the mechanisms of how exactly green space and biodiversity impact allergic and respiratory health remain unclear. Along with pollen, factors such as humidity, moulds and local climate conditions may also be implicated. Air pollution exposure is another concern for respiratory heath (Thurston et al., 2017). Yet, it is unlikely that air pollution biased the associations between green space and the health outcomes observed in our study, since further adjusting for residential NO_2 or PM_{10} levels did not substantially change the estimated associations. However, we cannot rule out that other air pollutants may play a role.

5. Conclusions

Data collected from nine different European paediatric cohorts was meta-analysed to investigate potential associations between current residential land cover and common respiratory and allergic childhood diseases. Our results indicate that living close to large green space areas, in particular coniferous forests, may increase the risk of developing wheezing, asthma and allergic rhinitis in children.

Our findings also support research showing that agricultural areas near children's homes may have a protective effect on respiratory health, while exposure to urban/grey space did not seem to impact the development of wheezing, asthma or rhinitis. Additional studies evaluating both the type and quality of green space and its use, as well as the interaction with air and soil pollution in relation to respiratory conditions, should be conducted in order to clarify the underlying mechanisms behind the adverse respiratory impacts associated with green space.

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Ethical approval

Ethical approval was not requested for this secondary analysis of pooled data from previous studies. In each of the original studies, ethical approval was obtained for each centre from the appropriate ethics committee, see the Online Supplement. All procedures have conformed to the principles embodied in the Declaration of Helsinki.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.108953.

Contributors

The corresponding author Eija Parmes and Cara Nichole Maesano conceived of the study. Eija Parmes was responsible for CLC exposure estimates. Giancarlo Pesce designed the statistical analysis plan and performed the statistical analyses. Cara Nichole Maesano and Giancarlo Pesce drafted the first version of the manuscript. All the authors

contributed in the collection of data in/from the original studies, discussion of the statistical analysis plan and interpretation of the results. All the authors critically reviewed and approved the final version of the manuscript. Eija Parmes had final responsibility for the submission of the publication.

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ORIGINAL RESEARCH

Body mass index and weight change are associated with adult lung function trajectories: the prospective ECRHS study

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ABSTRACT

Background Previous studies have reported an association between weight increase and excess lung function decline in young adults followed for short periods. We aimed to estimate lung function trajectories during adulthood from 20-year weight change profiles using data from the population-based European Community Respiratory Health Survey (ECRHS).

Methods We included 3673 participants recruited at age 20–44 years with repeated measurements of weight and lung function (forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1)) in three study waves (1991–93, 1999–2003, 2010–14) until they were 39–67 years of age. We classified subjects into weight change profiles according to baseline body mass index (BMI) categories and weight change over 20 years. We estimated trajectories of lung function over time as a function of weight change profiles using population-averaged generalised estimating equations.

Results In individuals with normal BMI, overweight and obesity at baseline, moderate (0.25–1 kg/year) and high weight gain (>1 kg/year) during follow-up were associated with accelerated FVC and FEV_1 declines. Compared with participants with baseline normal BMI and stable weight (±0.25 kg/year), obese individuals with high weight gain during follow-up had −1011 mL (95% CI −1.259 to −763) lower estimated FVC at 65 years despite similar estimated FVC levels at 25 years. Obese individuals at baseline who lost weight (<−0.25 kg/year) exhibited an attenuation of FVC and FEV_1 declines. We found no association between weight change profiles and FEV_1/FVC decline.

Conclusion Moderate and high weight gain over 20 years was associated with accelerated lung function decline, while weight loss was related to its attenuation. Control of weight gain is important for maintaining good lung function in adult life.

Key questions

What is the key question?

► Is weight change over a 20-year period associated with lung function trajectories in adult life?

What is the bottom line?

► Moderate and high weight gain over a 20-year period was associated with accelerated FVC and FEV_1 decline, while weight loss was related to its attenuation.

Why read on?

► This study, which is based on data collected as part of the multicentre prospective ECRHS study, reinforces the public health message that overweight and obesity have deleterious effects on respiratory health. However, these negative effects can be reversed by weight loss even later in adult life.

BACKGROUND

Lung function is a significant predictor of future morbidity and mortality in the general population.[1] Maintaining good lung function across adult life is important to prevent chronic respiratory diseases, which nowadays represent a serious public health problem around the world.[2] There is consistent evidence showing that overweight, obesity and weight gain in adulthood are detrimental to lung function, as described by the forced vital capacity (FVC) and/or forced expiratory volume in 1 s (FEV_1). Previous population-based and occupational cohort studies have shown that excessive weight gain in adulthood is associated with lower lung function levels and with an increased rate of lung function decline independently of age and

smoking status.[3–8] Another longitudinal study in healthy young adults (age range at baseline 18–30 years) showed that lung function was lower both with higher baseline body mass index (BMI) and with increasing BMI over a 10-year period.[9] Similarly, a population-based study of young adults (mean age at baseline 41 years) analysing the effects of changes in obesity status on lung function found that remaining or becoming obese accelerated lung function decline over an 8-year follow-up, while becoming non-obese was related to its attenuation.[10]

All these previous studies have had relatively short follow-up periods (up to 10 years) and most investigated this link only up to 50 years of age. This precludes a more comprehensive understanding of the role of weight change on lung function during adulthood and older life and supports the need for further studies with longer follow-up periods extending into late adult life. Understanding the effects of weight changes on lung function during adult life is of utmost importance given the epidemic levels of overweight and obesity globally.[11]

The European Community Respiratory Health Survey (ECRHS) is a large multicentre population-based study with available measures of weight, height and lung function at three time points over a 20-year period, as well as detailed information of sociodemographic and lifestyle factors from adults living across Europe and Australia.[12–14] Under the framework of the Ageing Lungs in European Cohorts (ALEC) consortium (www.alecstudy.org), we aimed to assess the lung function trajectories of adults of the ECRHS study according to different weight change profiles that combined BMI at baseline and weight change over a 20-year period.

METHODS

Study population

The ECRHS started in 1991–1993 (ECRHS I), when over 18 000 young adults aged 20–44 years were randomly recruited from available population-based registers (population-based arm), with an oversampling of asthmatics (symptomatic arm). Participants were followed up in 1999–2003 (ECRHS II) and 2010–2014 (ECRHS III) when they were aged 27–57 and 39–67 years, respectively. More details of the study design are available elsewhere.[12–14] In this analysis we included participants who had weight at ECRHS I and III and lung function and base covariates (sex, age, height and smoking status) at all three surveys (3673 participants from 26 centres in 12 countries) (see online supplementary figure S1).

Ethical approval was obtained from the ethics committees of all participating institutions and all participants provided informed written consent.

Lung function

Lung function was measured by spirometry at ECRHS I, II and III. Centres used different spirometers at ECRHS I and II, but almost all centres used the same spirometer at ECRHS III (see online supplementary table S1). In the three examinations, forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1), repeatable to 150 mL from at least two of a maximum of five correct manoeuvres that met the American Thoracic Society and European Respiratory Society recommendations,[15] were used as the primary outcomes. The FEV_1/FVC ratio was also analysed. In the present analysis, we used lung function measurements collected pre-bronchodilator. We also calculated lung function SD scores (z-scores) using the Global Lung Initiative (GLI) equation references,[16] and used these variables as secondary outcomes.

Weight change profiles

BMI was calculated by dividing measured weight (kg) by measured height (m) squared. We defined categories of BMI at ECRHS I (baseline) as 'underweight' (BMI $<20\,kg/m^2$), 'normal weight' ($20\,kg/m^2 \leq BMI < 25\,kg/m^2$), 'overweight' ($25\,kg/m^2 \leq BMI < 30\,kg/m^2$) and 'obese' (BMI $\geq 30\,kg/m^2$), as in previous ECRHS studies.[8] We computed weight change during follow-up as the difference between weight measured at ECRHS III and ECRHS I divided by the total time of follow-up (in years) and categorised it into stable weight, weight loss and weight gain. Since there are no standard references for weight change in adults, we used similar weight change categories as in a recent longitudinal long-term population-based study[17]: 'weight loss' (<-0.25 kg/year), 'stable weight' (± 0.25 kg/year), 'moderate weight gain' (>0.25 to ≤ 1 kg/year) and 'high weight gain' (>1 kg/year). We combined baseline BMI categories with weight change categories to classify participants in weight change profiles. This combined variable was used as the main exposure variable in the analysis.

Other relevant variables

Sociodemographic and other health data were collected using questionnaires. These included sex, age, age completed full-time education (<17 years; 17–20 years;>20 years), smoking status (never smoker; ex-smoker; current smoker), secondhand smoke exposure (yes; no) and asthma (yes; no). Current asthma was defined as having reported physician-diagnosed asthma and at least one of the following: asthma-like symptoms (wheeze, nocturnal chest tightness, attacks of breathlessness after activity/at rest/at night-time), asthma attacks, use of inhaled/oral medicines for breathing problems (in the last 12 months), or current use of inhalers, aerosols or tablets for asthma. Leisure-time vigorous physical activity was assessed at ECRHS II by asking participants how often and for how many hours per week they usually exercised so much that they got out of breath or sweaty. Participants were categorised as being active if they exercised with a frequency of two or more times a week and with a duration of about 1 hour a week or more, and non-active otherwise.[18] Finally, at ECRHS II participants reported if they presented any of the following long-term limiting illnesses: hypertension, heart disease, diabetes, cancer or stroke.

Statistical analysis

We used population-averaged generalised estimating equations (GEE) to estimate lung function trajectories from age 20 to 67 years (the full age range of the study sample) as a function of weight change profiles. Prior to stratifying models by weight change profiles, we tested the interaction between age, BMI at baseline and weight change, and we found that it was statistically significant for all lung function parameters (p value <0.01 for all models). All GEE models had the individuals as the clustering factor (to account for repeated lung function measurements at ECRHS I, II and III) and an unstructured within-cluster correlation. GEE models had FVC, FEV_1 or FEV_1/FVC as the outcome variables. Interaction terms between age (or age squared) and weight change profiles were entered to allow for different trajectories of lung function with ageing across weight change profiles. We entered sex as a fixed covariate and height, age, age squared, smoking status, current asthma and spirometer type as time-specific covariates. We also included an interaction term between smoking status and age (to account for a faster decline over time in smokers). We centred continuous variables at the mean (over the data from the three examinations) before modelling. Adjusted lung function over age was calculated by

setting continuous and categorical variables equal to the mean and proportion, respectively (calculated over the study sample).

In a secondary analysis we repeated the models using lung function z-scores instead of absolute lung function values. To assess whether estimated lung function trajectories differed by sex we tested for sex interactions (by including an interaction term between sex and weight change profiles) and we stratified final models by sex. We performed several sensitivity analyses to assess the robustness of the estimated lung function trajectories to various assumptions regarding confounding, change of spirometry devices or weight change categorisation (see online supplementary file).

All analyses were conducted following a complete case approach in Stata/SE 14.0 (StataCorp, College Station, Texas, USA).

RESULTS

Characteristics of the study sample

Compared with those not included in the present analysis (n=12 909), individuals who were included were slightly older, less likely to be current smokers, be exposed to secondhand smoke and had higher educational levels at ECRHS I, but they did not differ in terms of weight, BMI and lung function (see online supplementary table S2). Table 1 shows the main characteristics of the study sample (n=3673). Mean (SD) age of the study sample was 34.3 (7.1) years at baseline and 54.3 (7.1) years at the last follow-up. Approximately half of the study sample were women (53.3%) and 40% had completed full-time education when they were 20 years of age or older.

At baseline, 12% of the sample was underweight, 57% normal weight, 24% overweight and 6% obese. During follow-up almost 4% of the sample lost weight, 34% had stable weight, 53% had a moderate weight gain and 9% had a high weight gain. Table 2 shows descriptive statistics of the 16 weight change profiles identified. Almost 20% of the sample was classified in the weight change profile with baseline normal BMI and stable weight during follow-up. Out of the groups who lost weight during follow-up, obese participants at baseline were those who lost more weight over time (median −0.6 kg/year, P_{25}–P_{75} −0.9 to −0.4), while among those who experienced a moderate increase in weight, median weight gain was the same in the different categories of baseline BMI. Among those with high weight gain during follow-up, overweight and obese participants at baseline were those who gained more weight. Underweight participants who lost weight or had a high weight gain represented less than 1% of the study sample and therefore were excluded from further analyses.

Table 1 Characteristics of the study sample*

Characteristics	ECRHS I N (%) or mean (SD)	ECRHS II N (%) or mean (SD)	ECRHS III N (%) or mean (SD)
Symptomatic study arm	544 (14.8)	–	–
Women	1956 (53.3)	–	–
Age in years	34.3 (7.1)	43.0 (7.0)	54.3 (7.1)
Height in cm	170.6 (9.4)	170.3 (9.4)	169.4 (9.5)
Weight in kg	69.5 (13.5)	74.0 (15.1)	77.9 (16.1)
BMI			
Continuous, in kg/m^2	23.8 (3.7)	25.4 (4.3)	27.1 (4.9)
Underweight	453 (12.3)	222 (6.1)	119 (3.2)
Normal weight	2097 (57.1)	1676 (45.8)	1224 (33.3)
Overweight	892 (24.3)	1298 (35.5)	1481 (40.3)
Obese	231 (6.3)	461 (12.6)	849 (23.1)
Smoking status			
Non-smoker	1651 (45.0)	1576 (42.9)	1518 (41.3)
Ex-smoker	818 (22.3)	1119 (30.5)	1500 (40.8)
Current smoker	1204 (32.8)	978 (26.6)	655 (17.8)
Secondhand smoke exposure, yes	1939 (52.9)	1321 (36.1)	680 (18.6)
Current asthma, yes†	378 (10.5)	491 (13.8)	570 (16.2)
Age completed full-time education			
<17 years	675 (21.5)	–	–
17–20 years	1205 (38.4)	–	–
>20 years	1256 (40.1)	–	–
Physical activity. Active status‡	–	1363 (52.2)	–
Any long-term limiting illness, yes§	–	405 (17.1)	–
Lung function			
FVC (mL)	4516 (988)	4354 (980)	3964 (948)
FEV_1 (mL)	3702 (798)	3485 (790)	3006 (753)
FEV_1/FVC (%)	82.3 (6.9)	80.3 (6.5)	75.8 (6.5)
Lung function (z-scores)¶			
FVC z-score	0.01 (0.95)	0.02 (1.00)	−0.08 (0.94)
FEV_1 z-score	−0.01 (1.06)	−0.03 (1.08)	−0.34 (1.04)
FEV_1/FVC z-score	−0.06 (1.03)	−0.10 (1.00)	−0.48 (0.89)

*Some variables had missing values. Number of missing values for ECRHS I: 10 in secondhand smoke exposure, 78 in current asthma, and 537 in age completed full-time education. Number of missing values for ECRHS II: 18 in secondhand smoke exposure, 118 in current asthma, 1062 in physical activity and 1300 in any long-term limiting illness. Number of missing values for ECRHS III: 14 in secondhand smoke exposure and 163 in current asthma.
†Current asthma was defined as having reported physician-diagnosed asthma and at least one of the following: asthma-like symptoms (wheeze, nocturnal chest tightness, attacks of breathlessness after activity/at rest/at night-time), asthma attacks, use of inhaled/oral medicines for breathing problems (in the last 12 months), or current use of inhalers, aerosols or tablets for asthma.
‡Individuals were categorised as being active if they exercised with a frequency of two or more times a week and with a duration of about 1 hour a week or more.
§The following illnesses were considered: hypertension, heart disease, diabetes, cancer or stroke.
¶Lung function z-scores were derived using Global Lung Initiative 2012 equations.
BMI, body mass index; FEV_1, volume expired in the first second; FVC, forced vital capacity.

Associations between weight change profiles and lung function trajectories

To facilitate interpretation of results, the estimated trajectories of lung function by weight change profiles are presented separately for normal BMI, overweight and obese categories at baseline (figures 1–3). Among adults with baseline normal BMI, overweight and obesity, those with moderate and high weight gain during follow-up exhibited significantly steeper FVC decline than those with stable weight (Panels A, B and C in figure 1). Estimated differences in FVC at 25 and 65 years by weight change profiles (see online supplementary table S3) show that, in comparison with participants with baseline normal BMI and stable weight, baseline overweight and obese participants with high weight gain had lower estimated FVC at 65 years (−677 mL (95% CI −841 to −512); p<0.001 and −1.011 mL (−1.259 to −763); p<0.001, respectively) despite similar estimated FVC levels at age 25 (see online supplementary table S3).

In contrast to weight gain, obese (but not overweight or normal BMI) adults at baseline who lost weight during follow-up exhibited an attenuation of FVC decline (panel C in figure 1). We estimated that, at age 25 years, obese participants had lower FVC levels than normal BMI participants. However, obese individuals who lost weight during follow-up were estimated to have not significantly different FVC values at age 65 years than participants with baseline normal BMI and stable weight (see online supplementary table S3).

Table 2 Descriptive statistics of weight change profiles

Weight change profiles*		N (%)	Weight ECRHS I (kg) Median (P_{25}; P_{75})	Weight ECRHS III (kg) Median (P_{25}; P_{75})	Weight change during follow-up (kg/year) Median (P_{25}; P_{75})
Underweight	Weight loss	2 (0.1)†	55.5 (54; 57)	48.5 (45; 52)	−0.3 (−0.4; −0.3)
	Stable weight	167 (4.6)	53 (50; 56)	55 (51; 59)	0.1 (0; 0.2)
	Moderate weight gain	259 (7.1)	53 (50; 58)	65.3 (60; 70.4)	0.5 (0.4; 0.7)
	High weight gain	25 (0.7)†	52 (50; 57)	78 (74; 85)	1.2 (1.1; 1.5)
Normal BMI	Weight loss	38 (1)	63.5 (60; 74)	55 (52; 65)	−0.4 (−0.4; −0.3)
	Stable weight	715 (19.5)	64 (59; 72)	65.8 (60; 74)	0.1 (0.0; 0.2)
	Moderate weight gain	1164 (31.7)	65 (60; 72)	76 (70; 84)	0.5 (0.4; 0.7)
	High weight gain	180 (4.9)	66 (60; 72)	92.4 (86; 98)	1.2 (1.1; 1.4)
Overweight	Weight loss	52 (1.4)	80 (76; 87)	71 (66; 75.8)	−0.4 (−0.6; −0.3)
	Stable weight	291 (7.9)	79 (73; 85)	80 (73; 86.8)	0.1 (−0.1; 0.2)
	Moderate weight gain	454 (12.4)	80 (73; 86)	90.9 (84; 97.1)	0.5 (0.4; 0.7)
	High weight gain	95 (2.6)	79 (70; 85)	103 (96.4; 113.9)	1.3 (1.1; 1.5)
Obese	Weight loss	46 (1.3)	95 (87; 105)	85 (72; 93)	−0.6 (−0.9; −0.4)
	Stable weight	65 (1.8)	90 (85; 100)	92 (85; 101)	0.1 (−0.1; 0.1)
	Moderate weight gain	85 (2.3)	93 (87; 103)	105 (97.1; 114)	0.5 (0.4; 0.7)
	High weight gain	35 (1)	95 (85; 109)	125 (112; 135)	1.3 (1.1; 1.8)
Overall		3673 (100)	68 (59; 78)	76 (66; 87.3)	0.4 (0.1; 0.7)

*Weight change profiles were defined combining BMI at baseline and weight change during follow-up. BMI categories at baseline: underweight: BMI <20 kg/m^2; normal weight: 20 kg/m^2≤BMI<25 kg/m^2; overweight: 25 kg/m^2 ≤BMI <30 kg/m^2; obese: BMI ≥30 kg/m^2. Weight change was computed as the difference between weight measured at ECRHS III and ECRHS I divided by the total duration follow-up (in years). Weight change categories: weight loss: <−0.25 kg/year; stable: within ±0.25 kg/year; moderate weight gain: 0.25–1 kg/year; high weight gain: >1 kg/year.
†Not analysed further because of small sample size.

Supplementary figure S2 shows lung function trajectories for subjects with baseline underweight. In young adulthood, participants with baseline underweight had lower estimated FVC values than baseline normal BMI participants (see online supplementary figure S2). However, baseline underweight participants with stable weight during follow-up were estimated to have very similar FVC values at age 65 to participants with baseline normal BMI and stable weight.

We found very similar results for estimated FEV_1 trajectories (figure 2, online supplementary figure S2 and table S4). We found no evidence that FEV_1/FVC ratio trajectories were different by weight change profiles, except for two groups. Subjects with baseline underweight who had stable weight or moderate weight gain showed a steeper decline in FEV_1/FVC ratio than participants with baseline normal BMI and stable weight during follow-up (figure 3, online supplementary figure S2 and table S5).

Secondary analysis using lung function z-scores instead of absolute lung function showed similar results to the main analysis for all lung function parameters (see online supplementary figure S3). Stratification by sex showed that FVC and FEV_1 decline was steeper in men who gained weight than in

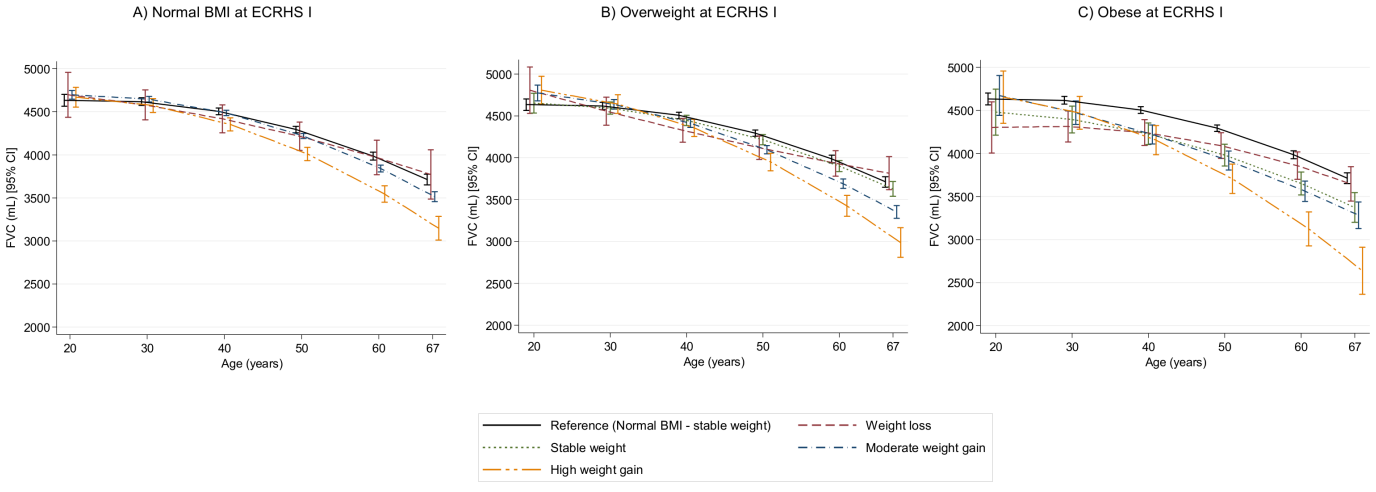

Figure 1 Estimated trajectories of FVC (in mL) decline by weight change profiles. The figure shows estimated FVC values and their corresponding 95% CI. Models are adjusted for sex, height, age, age squared, smoking status, an interaction term between smoking status and age, current asthma and spirometer type. Reference category: normal BMI at baseline and stable weight during follow-up. All graphs are presented with a 'jitter' (0.05) to avoid overlap of CI bars. BMI, body mass index; FVC, forced vital capacity.

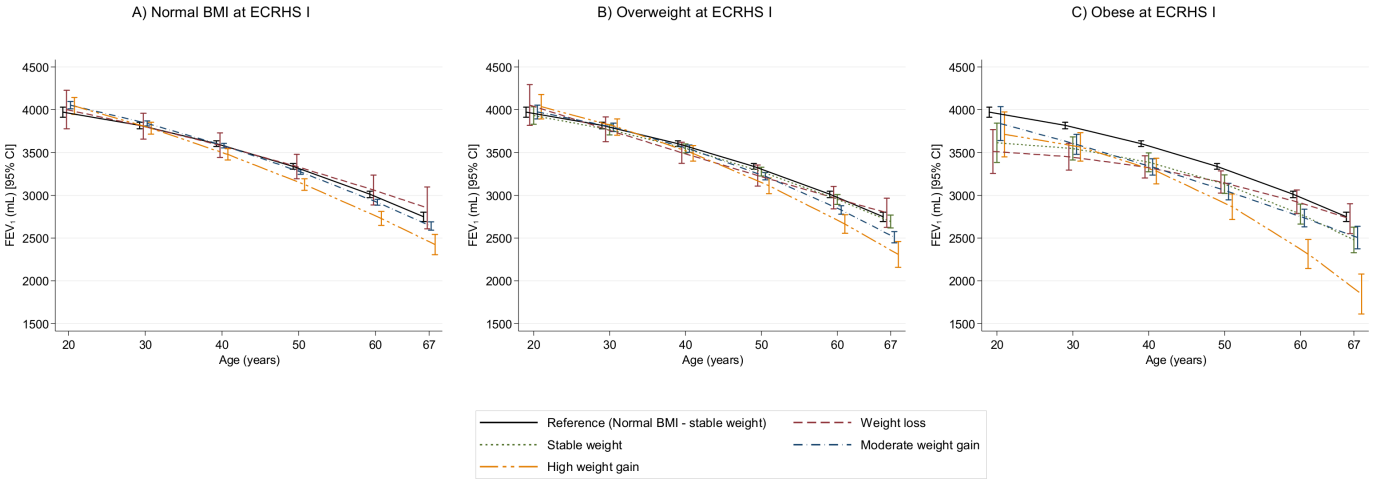

Figure 2 Estimated trajectories of FEV_1 (mL) decline by weight change profiles. The figure shows estimated FEV_1 values and their corresponding 95% CI. Models are adjusted for sex, height, age, age squared, smoking status, an interaction term between smoking status and age, current asthma and spirometer type. Reference category: normal BMI at baseline and stable weight during follow-up. All graphs are presented with a 'jitter' (0.05) to avoid overlap of CI bars. BMI, body mass index; FEV_1, forced expiratory volume in 1 s.

their female counterparts, particularly in the obese category (see online supplementary figure S4 and S5), but there was no difference with regard to the FEV_1/FVC ratio (see online supplementary figure S6). All sensitivity analyses showed very similar results (see online supplementary figure S7–S12). However, the lung function differences between the reference category and some overweight/obese weight change profiles were attenuated when the analyses were restricted to participants who reported to be non-smokers at all examinations and when additionally adjusting for physical activity, educational level and any long-term limiting illness.

DISCUSSION

In this population-based study we found that weight change over a 20-year period was associated with the rate of lung function decline in adulthood. Specifically, we found that: (1) in participants with baseline normal BMI, overweight and obesity in young adulthood, moderate and high weight gain during follow-up were associated with accelerated FVC and FEV_1 decline; (2) in participants with obesity in young adulthood, weight loss during follow-up was associated with attenuated FVC and FEV_1 decline; (3) in underweight participants in young adulthood, stable weight during follow-up was associated with an attenuation of FVC and FEV_1 decline; and (4) we found no evidence of an association between weight change and FEV_1/FVC ratio decline, with the exception of underweight participants with either stable weight or moderate weight gain, both of whom exhibited accelerated FEV_1/FVC ratio decline over follow-up.

Interpretation

Our findings that moderate and high weight gain accelerates FVC and FEV_1 decline and that weight loss attenuates it are consistent with previous research in young adults.[3–10] This demonstrates how weight changes can affect lung function until late adulthood. Our approach of combining baseline BMI categories with weight change over time let us distinguish the effects of different weight change profiles on lung function throughout

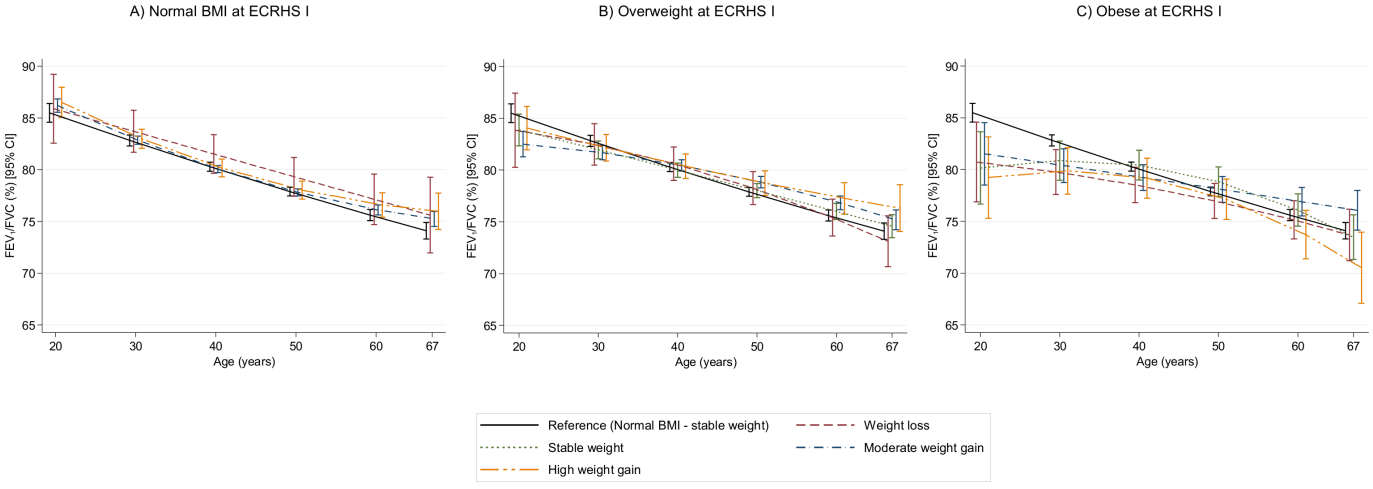

Figure 3 Estimated trajectories of FEV_1/FVC (%) decline by weight change profiles. The figure shows estimated FEV_1/FVC values and their corresponding 95% CI. Models are adjusted for sex, height, age, age squared, smoking status, an interaction term between smoking status and age, current asthma and spirometer type. Reference category: normal BMI at baseline and stable weight during follow-up. All graphs are presented with a 'jitter' (0.05) to avoid overlap of CI bars. BMI, body mass index; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

adult life. Two potential mechanisms have been proposed to explain the association of weight gain with accelerated lung function decline. First, weight gain can affect lung function through mechanical effects on lungs. Abdominal and thoracic fat mass are likely to reduce vital capacity by limiting the room for lung expansion during inspiration, in turn leading to expiratory flow limitation.[19] These mechanical effects may also explain the observed sex differences in relation to lung function decline, consistent with previous studies,[4 8 20] as men tend to accumulate more fat mass in the abdominal area than women.[21] Second, weight gain can impair lung function by inflammatory processes, as adipose tissue is a source of inflammatory mediators[22 23] that can damage lung tissue and reduce airway diameter.[24] Unfortunately, we did not have measures of chest compliance or markers of systemic inflammation related to obesity, and therefore we could not disentangle the mechanical effects of body mass on lung function from the inflammatory effects.

There are some potential mechanisms that can explain the association between weight loss and attenuation of lung function decline in obese subjects. First, it is possible that weight loss reverses the mechanical effects of overweight/obesity on the respiratory system allowing the recovery of lung function. Second, weight loss may relate to a reduction of inflammatory processes in the lung which in turn can help to attenuate lung function decline related to excessive weight. This hypothesis is supported by previous research showing that lung function decline associated with air pollution, which likely affects lung function via inflammation, could be attenuated with improvement of air quality.[25] Third, weight loss may be accompanied by improvement of metabolic alterations related to excess body weight, such as insulin dysregulation, high fasting glucose levels, hyperlipidaemia or systemic hypertension, which are also related to impaired lung function.[26 27] Fourth, the observed association between weight loss and attenuated lung function decline could be related to confounding by changes in lifestyle (eg, increasing physical activity or changing diet) that can follow awareness of the harmful effects of overweight/obesity. Indeed, quitting smoking and becoming physically active in adulthood has been related to better lung function levels and/or attenuated lung function decline.[8 18 28 29] Although we accounted for changes in smoking status during follow-up, levels of physical activity and presence of long-term limiting illness that could be accompanied by metabolic alterations (hypertension, heart disease, diabetes, cancer or stroke) at ECRHS II in sensitivity analyses, we did not have information on physical activity or diet at baseline. Further studies with repeated measures of lifestyle factors and indicators of metabolic dysregulation associated with weight changes are needed to disentangle the mechanisms underlying the association of weight loss and attenuated lung function decline.

We also found that stable weight during follow-up in individuals underweight in young adulthood was associated with attenuated FVC and FEV_1 decline, while those with baseline underweight and moderate weight gain had a parallel FVC and FEV_1 decline to individuals with baseline normal BMI in late adulthood. These findings contrast with results of a previous longitudinal study showing that increasing BMI in initially thin adults (aged 18–30) was associated with lung function improvement over 10 years.[9] This inconsistency could be related to differences in the definition of weight gain (ie, the use of BMI gain vs weight change) and to a different baseline age range. The relationship between weight change and lung function has received little attention in healthy underweight individuals, so further research is needed to understand the effects of weight change in underweight individuals and their underlying mechanisms.

In the present analysis we did not observe statistically different FEV_1/FVC ratio trajectories by weight change profiles, except for underweight subjects with either stable weight or moderate weight gain during follow-up, both of whom exhibited a faster FEV_1/FVC decline over follow-up. The observed associations in underweight subjects are in line with findings of one previous study in healthy adults[9] and allow us to hypothesise that underweight subjects could be more susceptible to the development of airflow limitation with ageing. Also, the lack of association of weight gain with the FEV_1/FVC ratio in the present analysis is in line with previous studies showing that the FEV_1/FVC ratio is normal in overweight and obese individuals.[19] The lack of association of weight gain with the FEV_1/FVC ratio could be attributed to the fact that both FVC and FEV_1 declines were accelerated with weight gain, which could lead to a null net effect on the ratio of these two measures (as both denominator and numerator were equally affected). This pattern suggests that weight gain is likely to be related to a restrictive pattern characterised by a reduction of lung volumes with no effect on airflow limitation. This hypothesis is supported by previous evidence showing that obesity is more likely to be associated with a restrictive ventilatory pattern than an obstructive one.[30]

Strengths and limitations

A strength of the current study is the long follow-up (up to 20 years) and the width of age distribution covering early to late adulthood. The population-based nature of the ECRHS and broad geographical representation of participants (26 centres in 12 countries in Europe and Australia) support external validity of our results. Finally, we had lung function measures at three time points, which allowed us to estimate lung function trajectories.

A limitation of this study is the use of total body weight as the main exposure. Although total body weight has been largely used in epidemiological studies as a marker of overweight and obesity, it is limited by its inability to distinguish between fat and muscle mass, which vary with age and sex[31 32] and could have different effects on lung function, as previously shown in children.[33] Also, we defined weight change categories using only weight measures at baseline and last follow-up to capture 'stable' weight change patterns and facilitate the interpretability of our results. Of note, the correlation between individual weight change per year (taking into account three weight measurements) and the weight change variable used in our analysis was 0.998, which justifies our approach. However, we recognise that our approach precludes us from determining how long it takes for a change in weight to affect lung function decline. Given the lack of standard references for weight change in adults, we categorised weight change based on a previous longitudinal study,[17] limiting the interpretation of our findings to our definition of 'stable weight' (±0.25 kg/year). However, the results were very similar when repeating our analysis using a wider category for 'stable weight' (±0.50 kg/year), suggesting that our findings are robust even with a less restrictive definition of 'stable weight'. Our results may also be affected by selection bias, as participants were more likely to be highly educated and less likely to be current smokers or to be exposed to secondhand smoke than those lost to follow-up. Because these factors have been previously associated with lung function, our associations could be underestimates of the true associations in the general population. Although we accounted for a wide range of confounders, our results could be affected by potential residual confounding by, for example, dietary intake, which affects both body weight and lung function, as the available data on diet were limited to a small subset of the study sample at ECRHS II and III. Moreover, the spirometers used for

lung function assessment were changed in some centres, which could have led to systematic differences inherent in lung function measurement that may differ by age and height.[34] However, when we adjusted our analysis for spirometer type and when we replicated the analyses using lung function values corrected for change in spirometer we obtained consistent results. Finally, we used three repeated measures of lung function from a sample aged 20–44 years (mean (SD) age 34.3 (7.1) years) at baseline and 39–67 years (mean (SD) age 54.3 (7.1) years) at the last follow-up to estimate lung function trajectories throughout adulthood. However, few participants were aged around 20 years at baseline and around 67 years at the last follow-up, and in consequence the models had fewer observations at the age ends than between 30 and 60 years, where most of the observations were.

CONCLUSION

In conclusion, this prospective population-based study shows that moderate and high weight gain over a 20-year period was associated with accelerated lung function decline in adulthood, while weight loss was related to its attenuation. Our findings, together with the existing literature, reinforce the public health message that overweight and obesity have deleterious effects on health, including respiratory health. However, the negative effects of overweight and obesity on lung function can be reversed by weight loss even later in adult life. Therefore, public health policies that promote healthy lifestyles and body weight may be important for maintaining good lung function in adult life.

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Article

Formaldehyde, Oxidative Stress, and FeNO in Traffic Police Officers Working in Two Cities of Northern Italy

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Abstract: Personal air formaldehyde (air-FA) was measured as risk factor of airways inflammation and oxidative stress (SO) induction. Overall, 154 police officers were enrolled from two differently urbanised Italian cities, Turin and Pavia. Urinary F2t-isoprostane (15-F2t-IsoP), a prostaglandin-like compound, was quantified as a biomarker of general OS in vivo and fractional exhaled nitric oxide (FeNO) was measured for monitoring local inflammatory processes. Urinary cotinine was quantified as a biomarker of tobacco smoking exposure. Traffic police officers living in Turin showed an increased level of log air-FA ($p < 0.001$), equal to +53.6% ($p < 0.001$). Log air-(FA) mean values were 3.38 (C.I. 95% 3.33–3.43) and 2.84 (C.I. 95% 2.77–2.92) in Turin and Pavia, respectively. Log (air-FA) was higher in "outdoor workers" (3.18, C.I. 95% 3.13–3.24, $p = 0.035$) compared to "indoor workers", showing an increase of +9.3%, even controlling for sex and city. The analyses on 15-F2t-IsoP and FeNO, both adjusted for log air-FA, highlighted that OS and inflammation were higher (+66.8%, $p < 0.001$ and +75%, $p < 0.001$, respectively) in Turin traffic police officers compared to those from Pavia. Our findings suggest that even low exposures to traffic-related emissions and urbanisation may influence both general oxidative stress levels and local inflammation.

Keywords: public health; traffic police officers; formaldehyde; 15-F2t-isoprostane; FeNO

1. Introduction

Due to their toxicological and/or carcinogenic properties, some volatile organic compounds (VOCs), aromatic hydrocarbons and carbonyls have increasingly gained attention in the international scientific community dealing with public health issues [1–3]. In the last decades, researchers, interested in non-communicable diseases and prevention, focused on the interaction between the aforementioned chemicals and human health, especially in preventive terms [4–6]. Formaldehyde (FA), the simplest among the aldehydes, is an ubiquitous pollutant in living and occupational environments [7].

FA may directly originate from natural sources, from several anthropogenic activities and from indirect product via the photochemical oxidations of hydrocarbons in the atmosphere [8,9]. FA has been detected in several occupational settings involving wood-based materials, laminates, paints and urea-based resins [10] and is used as disinfectant and preservative in hospitals and in pathology units [11]. According to the International Agency for Research on Cancer (IARC) [12] and the United

States Environmental Protection Agency (US EPA) [13], FA is classified as a human carcinogen. FA-related exposures are connected to many other health effects, such as eye and respiratory tract irritations, allergic contact dermatitis and bronchial asthma [12,14].

Some previous studies investigated the influence of air pollution and FA on inflammation and Oxidative Stress (OS) induction [11,15,16] and on DNA toxicity [17,18]. Conversely, data on personal exposure to FA and OS induction in urban air settings are still lacking. Exposure to environmental pollution may lead to OS and inflammation, which in turn are able to determine biological outcomes and health impairments [19]. Exposure to FA may enhance oxidant species and impair the antioxidant system leading to OS. This latter can result in structural and enzymatic changes in organs [20], cytotoxic effects [21] and carcinogenic processes.

Raffic police officers may undergo long-term exposures to traffic-related pollutants [17,18]. For this reason, they can represent a population model suitable to describe some potential risky conditions for health in urban settings. Therefore, this population was considered suitable to assess the exposition to outdoor and indoor FA and some potentially consequent health effects such as OS and airway inflammation.

Although air-FA is usually higher indoors, the exposure in outdoor environments may considerably contribute to the human daily intakes of FA, the concentration of which is extremely variable in relation to many aspects such as the demographic density, the urban conformation and traffic management, the characteristics and the dimension of the human activities. In this respect, we speculated that the more urbanised cities may be considered greater sources of air-FA, representing a potential additional risky condition for health, compared to smaller cities.

This cross-sectional study aims to evaluate the role of FA exposure and the urbanisation level on OS induction, quantified by urinary 15-F2t-Isoprostane (15-F2t-IsoP), and on airway inflammation, measured by the fraction of exhaled nitric oxide (FeNO). It is notable that 15-F2t-IsoP is a reliable and sensitive biomarker of OS, used in several other epidemiological studies [10,11,16]. FeNO is measured in a non-invasive way and is a biomarker of eosinophilic airway inflammation, useful in monitoring inflammatory processes due to air pollution exposure [20].

A sample of traffic police officers from Turin and Pavia, two north-western Italian cities with different levels of urbanisation, was enrolled.

2. Materials and Methods

In total, 154 traffic police officers were enrolled in this study from November 2015 to March 2016; 109 of them were from Turin and 45 from a smaller town, Pavia. Turin (886,837 inhabitants) is located 239 m above sea level (a.s.l.), and has a population density per km^2 equal to 6813, while Pavia (72,612 inhabitants) is located 77 a.s.l. and has a population density equal to 1155 per km^2. Both cities are located in the north-western part of Italy, in the Po Valley (Figure 1), and are approximately 160 km from each other. The Po Valley (470,000 km^2) is one of the largest European plains, and is relatively homogeneous in terms of lifestyle, social and working conditions. In ecological terms, its climate is continental and, due to frequent thermal inversion episodes, vertical and horizontal air exchanges are more difficult compared to other areas of Europe, thus the air quality is poor [22].

The workers eligible to participate in the study were those working as traffic police officers in the urban areas of Turin and Pavia, completing different outdoor tasks such as traffic management (for a maximum of 2 h per working shift) as well as those working indoors. The invitation to participate was addressed to workers of both sexes and no other exclusion criteria were considered. In total, 154 traffic police officers participated by providing their written informed consent. The epidemiological sample did not involve the enrolment of subjects as controls. This is because the purpose of the study, as previously mentioned, was to compare two urban realities and different tasks, performed indoors and outdoors, without focusing on exposed and unexposed subjects.

On the day set for sampling, the subjects wore during the working shift (8 h) a personal air-sampler (Radiello®) to measure air-FA [11] (https://www.restek.com/pdfs/radiello-manual.pdf). At the end of

the working shift, the traffic police officers filled out a questionnaire and provided a spot of urine for the quantification of 15-F2t-IsoP and cotinine, as biomarkers of OS and tobacco smoking exposure, respectively. Moreover, two groups of pulmonologists measured, at individual level, FeNO as a marker of airway eosinophils inflammation.

This study obtained ethical approval in accordance with the Helsinki Declaration of 1975 ("Fondazione I.R.C.C.S. Policlinico San Matteo, Pavia" protocol number: 20130000718).

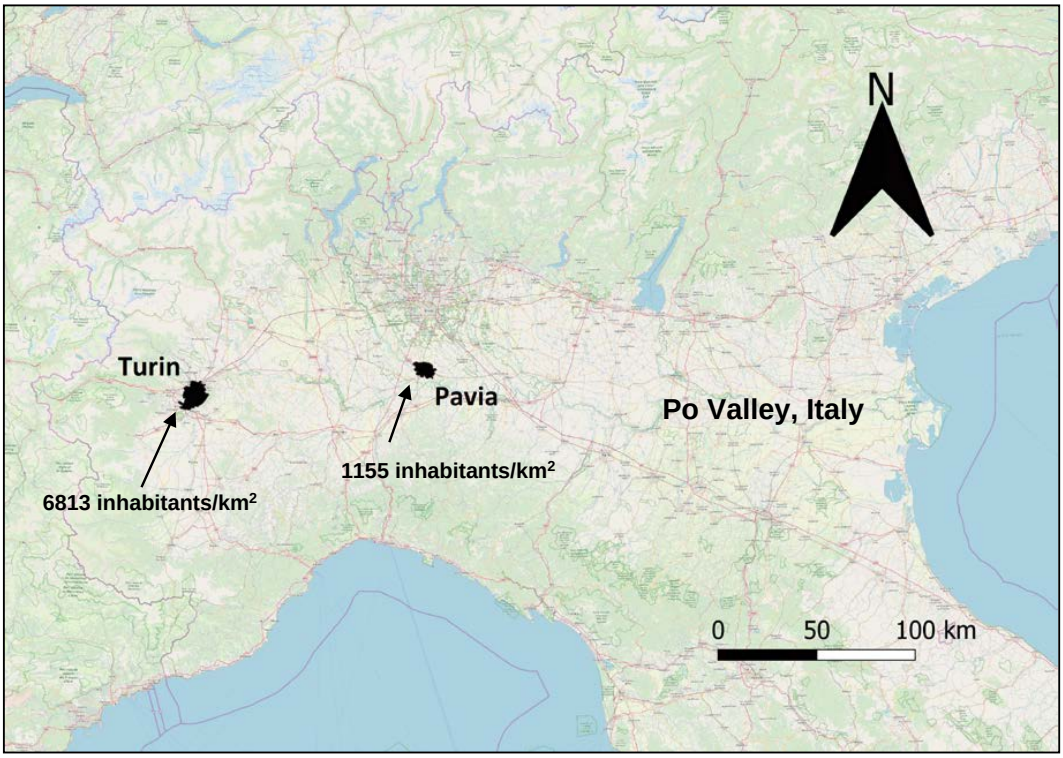


Figure 1. Map showing study area. The human density of the two cities, about 160 km apart, is reported as inhabitants per square kilometer.

2.1. Questionnaire

The same skilled person administered the questionnaire to each subject in the Turin and Pavia headquarters of the traffic police officers, collecting details about anthropometric characteristics, job tasks and location, years of service and potential confounders in OS induction such as diet, physical activity and tobacco smoke habits.

2.2. Personal Air-FA

Air-FA samples were collected for a whole working shift (8 h), using passive personal air samplers working with the radial symmetry (Radiello®), clipped near the breathing zone of the subject. The personal air samplers were equipped with a specific sorbent tube containing a 35–50 florisil mesh coated with 2,4-dinitrophenylhydrazine (DNPH). DNPH reacts with FA yielding 2,4-dinitrophenylhydrazone, which was subsequently quantified by high performance liquid chromatography (HPLC) according to the NIOSH method No. 2016 [23]. Briefly: the sampling rate value Q for Radiello® at 298 K (25 °C) and 1013 hPa is 99, the linearity range in $\mu g/m^3$ is 1000 ÷ 4,000,000, the limit of quantitation is $\mu g/m^3 = 0.1$, and uncertainty is at 2σ%, is 13.8.

The following materials are required to proceed with desorption: HPLC or spectroscopy grade acetonitrile, class A volumetric pipette, capacity 2 mL, micropore filter membranes, porosity 0.45 µm, solvent resistant. Procedure: 2 mL acetonitrile were introduced directly in the cartridge tube, recap and stir from time to time for 30 min. The resulting solution was filtered and kept well capped until analysis

time. When the analysis was delayed, the solution was stored at 4 °C. Materials for instrumental analysis: reverse phase C18 HPLC column, length 150 mm, 4.6 mm diameter, 5 μm packing particle size. The HPLC apparatus was capable of elution gradient and UV detection. Procedure: the detector was set at a wavelength of 365 nm. Between 10 and 50 μL of the solution was injected and eluted at flow of 1.9 mL·min−1. An isocratic elution was done with acetonitrile/water 38:62 *v*/*v* for 10 min, up to acetonitrile/water 75:25 *v*/*v* in 10 min, and reverse gradient to acetonitrile/water 38:62 *v*/*v* in 5 min. Finally, the detection limit was calculated as the sample concentration, providing a signal-to-noise ratio of 3. The quantification limit was considered to be twice when compared to the detection limit: 0.10 μg mL^{-1} and 0.05 μg mL^{-1} respectively. The CV values were <5%.

2.3. Biological Analyses

Urinary samples were collected at the end of the working shift, divided into aliquots, and stored at −80 °C until analyses. In order to normalise the individual excretion rate of all biological parameters, the first aliquot was used to quantify the urinary creatinine concentration by the Kinetic Jaffé method [24]. Since active and passive tobacco smoke exposure may exert its pro-oxidant effect acting as a potential confounder, urinary cotinine was measure in another aliquot, using a specific ELISA kit (Abnova Corporation, Jhongli, Taiwan) following the manufacturer's instructions. Finally, the 15-F2t-IsoP was quantified by the ELISA technique following the manufacturer's instructions [16].

2.4. FeNO Measurements

FeNO was measured in accordance with the American Thoracic Society and European Respiratory Society recommendations [25], by the CLD 88 chemiluminescence analyser (Eco Medics, Durnten, Switzerland), at an exhaled flow of 50mL/s and given in part per billion (instrument limit values ranging between 0.1–5000 ppb). The same group of pulmonologists performed the measurements in the Turin and Pavia traffic police officer headquarters. Participants were requested to refrain from smoking, eating, drinking and doing strenuous exercise for one hour prior to the measurement. The instrument was turned on at least 15 min prior to use and set for a 10 s inhalation. Each subject underwent measurement after receiving detailed explanations by the personnel. A nearby mirror helped participants in exhaling at the correct speed. All subjects were in sitting position and were asked to empty their lungs through a single long exhalation and to exhale slowly and steadily into the mouthpiece. If the participants were unable to complete the test at the first attempt, more attempts, no more than nine per person, were repeated and recorded.

2.5. Statistical Analysis

Descriptive analyses were carried out with the chi-square test for categorical variables (gender, job duties, cities), and the t-test or Mann–Whitney U-test for quantitative parameters (age, BMI, FeNO, cotinine, 15-F2t-IsoP, and FA), as appropriate. Correlation analyses were performed using the non-parametric Spearman's test to investigate the correlations between the biomarkers of OS and tobacco smoke exposure (15-F2t-IsoP and cotinine) and of OS and personal air-FA (15-F2t-IsoP and FA). A logarithmic transformation (Log-e) was performed for all the variables that showed a non-normal distribution (15-F2t-IsoP and FA); the normality of the distribution was tested using the Shapiro–Wilk test. Multiple linear regression (MLR) models were used to test the relationship and strength of the association between the dependent variable, independent variable and covariates or confounders (sex, tobacco smoke, sampling location, and job duties, depending on the specific model). MLR is an extension of ordinary least squares (OLS) regression involving more than one explanatory variable. MLR has been used to check the association between dependent and independent variables, mainly based on their approximately linear relationship and on the absence of collinearity among the covariates. The most parsimonious model has been selected, comparing several models that included a different set of variables, selected by a stepwise method. All categorical variables included in the model were dichotomous (0–1).

Moreover, the analysis on predictive margins was performed to deeply investigate the influence of job duties on FA exposure between the two locations, the difference of 15-F2t-IsoP between Turin and Pavia, controlling for personal air-FA and FeNO variation among venues controlling for both FA and cotinine. The level of significance was set at $p \leq 0.05$ (two-tailed) for all tests. All analyses were performed using STATA SE v14.2 (Stata Corp, College Station, TX, USA).

3. Results

Table 1 reports the general details of the epidemiological sampling between the two cities. Although more police officers were sampled in Turin (71%) than in Pavia (29%), no differences were observed in the distribution of age, BMI, gender and job duties (carried out indoors and outdoors) between the two cities. As shown in Table 2, the mean values of urinary 15-F2t-IsoP and FeNO are significantly different across locations (both $p < 0.001$), as well as personal exposure levels of air-FA ($p < 0.001$). Conversely, urinary cotinine shows similar distributions among subjects working in the two sampling locations, highlighting that both considered populations show similar smoking habits.

Table 1. Numerical and percentage characteristics of subjects. The statistical differences between the two cities are reported. Age: Mann–Whitney (M–W) U-test, BMI t-test; gender and job duties: X-square test.

		Turin $N = 109$ (71%)	Pavia $N = 45$ (29%)	p-Value	Overall $n = 154$
Age (years)		45.5 ± 7.7	46.6 ± 7.6	0.390	45.8 ± 7.7
BMI (kg/m^2)		24.8 ± 3.7	24.1 ± 3.2	0.236	24.6 ± 3.6
Gender	**Females**	50 (46)	16 (36)	0.153	66 (43%)
	Males	59 (54)	29 (64)		88 (57%)
Job Duties	**Indoor**	59 (54)	26 (58)	0.765	85 (55%)
	Outdoor	50 (46)	19 (42)		69 (45%)

Table 2. General description of environmental and biological measurements presented as subgroups by city. The statistical differences between the two cities are reported (M–W U-test).

	Turin	Pavia	p-Value	Overall
FeNO (ppb)	37.7 ± 28.3	33.6 ± 48.4	<0.001	36.5 ± 35.2
15-F2t-IsoP (ng/mg of Creatinine)	9.1 ± 7.3	4.1 ± 2.1	<0.001	7.7 ± 6.7
FA (μg/m^3)	30.9 ± 9.8	17.5 ± 4.7	<0.001	27.0 ± 10.5
Cotinine (ng/mg of Creatinine)	15.4 ± 22.8	14.9 ± 28.9	0.641	15.4 ± 23.4

As depicted in Figure 2, air-FA and 15-F2t-IsoP are significantly and positively correlated (Spearman's rho = 0.241, $p = 0.003$). The MLR model, adjusted for job duties and sex, shows that traffic police officers working in Turin have a significantly higher level of log (air-FA), 53.6% more than workers in Pavia ($p < 0.001$). In particular, the two log (air-FA) mean values are 3.38 (C.I. 95% 3.33–3.43) in the traffic police officers working in Turin and 2.84 (C.I. 95% 2.77–2.92) in the traffic police officers working in Pavia. Moreover, controlling for sex and city, log (air-FA) is significantly higher in traffic police officers who carried out their job duties mostly outdoors (3.18, C.I. 95% 3.13–3.24, $p = 0.035$), with an increase of 9.3% compared to traffic police officers working indoors (Figure 3A). The analyses of the OS biomarker highlight that, controlling for log (air-FA), the levels of 15-F2t-IsoP remain significantly higher (+66.8%) in traffic police officers working in Turin, compared with those employed in Pavia ($p < 0.001$) (Figure 3B). Furthermore, the MLR model adjusted for the level of cotinine and for air-FA exposure shows that traffic police officers working in Turin have significant higher levels of FeNO (+75%) compared to those working in Pavia ($p < 0.001$) (Figure 3C). Finally,

FeNO is negatively correlated with urinary cotinine, thus with tobacco smoke exposure. Although not significantly ($p = 0.07$), this tendency is consistent with previous studies [26–29].

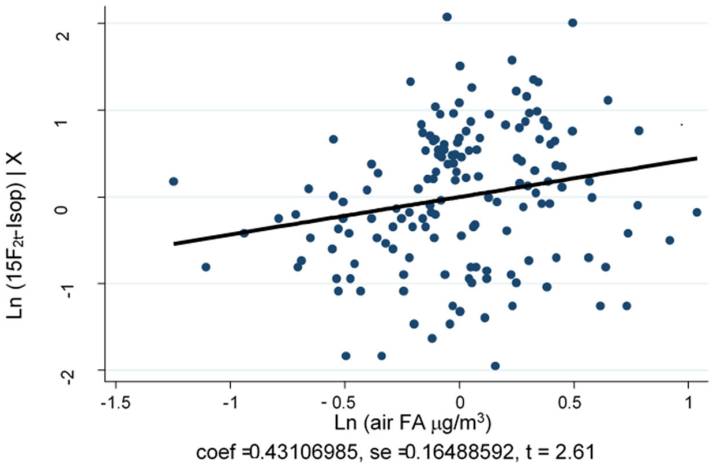
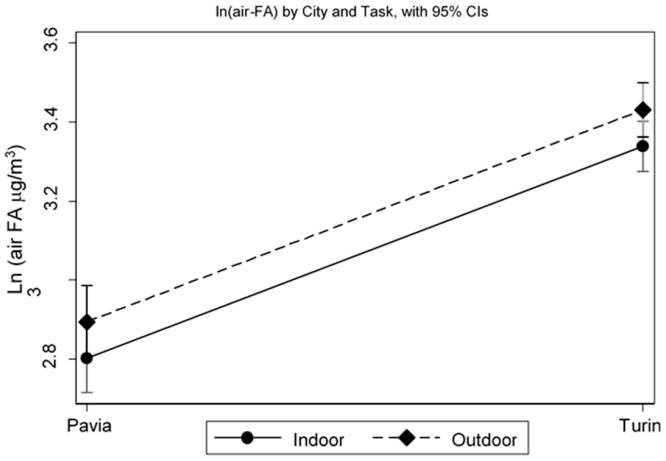
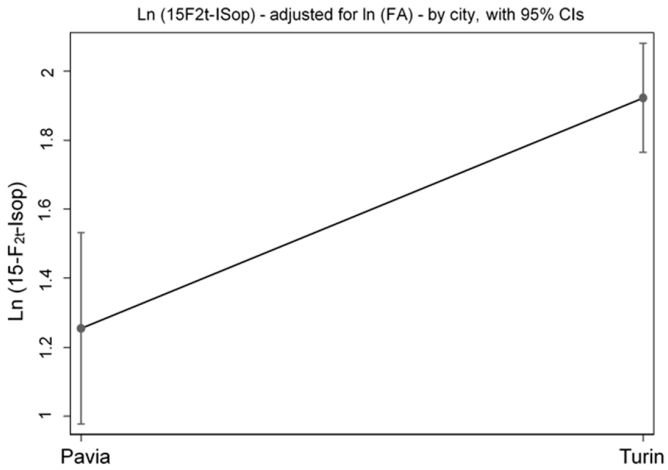


Figure 2. Correlation between Air-formaldehyde (FA) and 15-F_{2t}-IsoP. The correlation is significant and positive (Spearman's rho = 0.241, $p = 0.003$).

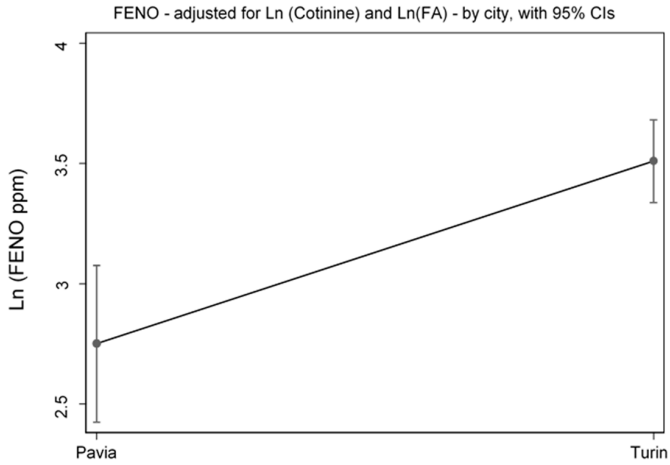


(**A**)



(**B**)

Figure 3. *Cont.*



(**C**)

Figure 3. MLR model. Log (air-FA), in Turin and in Pavia, indoors and outdoors (**A**), Log (15 F2t-IsoP) (**B**) and log (FeNO) (**C**).

4. Discussion

Air pollution is a matter of concern for human health due to its potential toxicological and/or carcinogenic effects on humans. Long-term exposures to air pollution, although in low doses, are recognised risk factors for the onset or exacerbation of several diseases, including respiratory infections and inflammations, cardiovascular impairments and cancer [30–32]. Among airborne VOCs, FA gained attention because of its toxicological and carcinogenic properties and for its primary and secondary origin in urban ambient air [12]. FA levels usually range between 1 and 20 μg/m^3 in urban air [33,34], with measurements that are usually higher in personal air compared to those measured by fixed samplers. This difference may depend on the complexity of the scenario described by the personal air-samplers. In fact, they are able to quantify a more comprehensive exposure accounting for all locations visited by the subjects.

In indoor environments, sources emitting FA are numerous, and air exchanges and dilutions processes are scarcer than in outdoor environments [8,35,36]. However, this study analysed a specific scenario of exposure, because the traffic police officers spend most of their working shift outdoors, differently from many other workers. Specifically, exposure to air-FA of traffic police officers is significantly higher in Turin than in Pavia, the smaller town. This result may depend on the different level of urbanisation.

Limiting the observation to outdoor pollution, the Po Valley is one of the most polluted areas in Europe. The orographic barriers enclose this area, characterised by weak winds and depressed surface; these factors provide less chances of air dilution processes, keeping the concentrations of air pollution high [37–40].

As above-mentioned, Turin and Pavia have different population densities and different air-FA concentrations. Besides the general ambient air quality in the two different urban contexts, this result emphasises the importance of traffic-related air-FA exposure. Additionally, the use of personal air-samplers has adequately characterised the individual exposures, allowing deeper investigations about the potential biological effects and health risks for the workers who participated in the study.

The analyses of general OS and local airways inflammation confirm that air pollution, tobacco smoking and air-FA exposures are able to determine biological effects, reflecting the interaction between environment and human exposure. Furthermore, our results show that the OS biomarker is significantly and positively correlated with both air-FA exposures and urinary cotinine (both $p < 0.001$). Interestingly, 15-F2t-IsoP is higher in traffic police officers employed in Turin if compared to those working in Pavia, even controlling for FA exposures. The model adjusted for cotinine, diet, and BMI

did not reach statistical significance, suggesting that other factors, not considered in this study, could contribute to determining an increase of 15-F2t-IsoP, which remains aspecific, besides its reliability in assessing OS status. Nevertheless, the modification of the behaviour of isoprostane only for FA appears to be a finding of considerable importance, even if the correlations are weak.

The traffic police officers of the two cities report a FeNO mean value ranging from 25 to 50 ppb. In particular, higher FeNO levels were recorded in the traffic police officers of Turin. Nevertheless, no correlations were found between FeNO and FA, 15-F2t-IsoP and cotinine, respectively. Unlike other studies [41,42], our findings do not show higher FeNO levels in men, nor in older subjects. FeNO levels measured in our study were classified as "intermediate FeNO", according to ATS [43] guidelines, which state that cautious interpretation is required at these values, considering the clinical contexts and the individual characteristics of the subjects (e.g., age and gender).

Differently from other authors [42], no relation has been found between FeNO and allergies. Finally, adjusting for sampling locations, FeNO shows a negative association with cotinine levels, although not significant ($p = 0.07$). This result is consistent with previous studies [41,44,45], since tobacco smoke is a recognised factor in the downregulation of the nitric oxide synthase, resulting in lower FeNO levels [42]. At this concern, several mechanisms have been proposed to explain the influence of tobacco smoke on FeNO levels. Nitrogen oxide in respiratory tract originates from inducible NO synthase (iNOS), regulated by interferon gamma (IFN-γ). Tobacco smoke may interfere in this pathway by reducing IFN-γ or directly inducing oxidative processes in the airways leading to the scavenging of NO [44].

5. Conclusions

In conclusion, our results suggest that even low doses and relatively small differences among environmental air-FA exposures may contribute to OS induction and airways inflammation. Traffic-related emissions and population densities play an important role in the air pollution exposure of workers who carry out their job duties mostly outdoors, as the traffic police officers do. These latter are exposed to a wide range of airborne pollutants, many of which are capable of inducing oxidative stress and airways inflammation.

Personal exposure measurements, quantified by personal air-samplers, are more accurate than those measured by fixed samplers and better represent the air quality, especially in complex environments such as the urban air. In other words, fixed sampling stations of the Regional Agency for the Protection of the Environment (A.R.P.A.) were not able to accurately describe the individual exposure to air pollutants during the working shift. There are five fixed sampling stations in Turin and two in Pavia, while the personal samplers have been worn by each worker ($n = 154$). Finally, FA is not regularly sampled by fixed sampling stations of A.R.P.A.

A limitation of this study is that only the exposure to FA was measured. However, FeNO, OS, tobacco smoke and air-FA were quantified by standardised and reproducible methods; thus, the comparison between two different polluted cities, located within the same geographic area in the south part of Europe, underlines the role of the urbanisation as an environmental human health risk factor. Future perspectives in this sense may drive further analyses such as the "greyness" quantification in urban environment, in order to better understand how the urbanisation interacts with human exposure and the consequent human health risk.

The traffic police officers working in Turin displayed higher levels of all measured biomarkers, with the exception of cotinine, a biomarker of exposure to tobacco smoke. Overall, these data highlight the influence of the living environment on the biological parameters, showing its influence on both local and general OS and inflammation.

Further investigations could consider a multiple airborne pollutants analysis, also a multi-site sampling for more comprehensive comparisons on the urbanisation levels.

In the future, additional analyses on urban settings, such as greyness/greenness quantification [46], may be helpful in driving new preventive strategies related to urban management, for outdoor workers and the general population.

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Article

Bisphenol A, Tobacco Smoke, and Age as Predictors of Oxidative Stress in Children and Adolescents

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Abstract: Objectives. The purpose of this study was to investigate bisphenol A (BPA) and its role in the induction of oxidative stress and confirm the same for tobacco smoke. Methods. A total of 223 young, healthy students (7–19 years old) were recruited in Chivasso, Italy. A spot of urine of each subject was analyzed to quantify BPA, cotinine, and 15F2t-isoprostane. Results. BPA showed a slight increase of concentration proportional with increasing age, even though the 11–14 years age group had slightly lower results, inducing a V-shape. The same trend was observed for 15F2t-isoprostane and cotinine. The result of piecewise linear robust regression shows a break point of the effect of BPA on 15F2t-isoprostane at 6 ng/mg CREA ($p < 0.001$). At higher levels, 15F2t-isoprostane shows an exponential increase by more than threefold for each one-log unit of BPA. An increase of oxidative stress due to BPA was observed, but only from 6 ng/mg of CREA up. Passive tobacco smoke is also able to induce an increase in oxidative stress. Conclusion. Prevention against BPA and passive tobacco smoke represents an important tool for promoting the highest health standard.

Keywords: oxidative stress; adolescents; passive tobacco smoke; BPA; public health

1. Introduction

Due to its endocrine disruptor properties and widespread presence in the human life environment, bisphenol A (BPA) is an important topic in terms of public health. BPA, whose IUPAC (International Union of Pure and Applied Chemistry) name is 2, 2-bis (4-hydroxyphenyl) propane (CASRN: 80-05-7), is a synthetic organic compound with a relatively short life [1]. The monomeric form of BPA is used in plastic food contact materials, in accordance with Commission Regulation (EU) No. 10/2011/EU on plastic materials coming into contact with foodstuffs. Furthermore, based on the precautionary principle, in 2011, the European Commission introduced the Implementing Regulation (EU) No 321/20118, which placed a restriction on the use of BPA in the manufacture of infant feeding bottles.

According to the European Food Safety Authority (EFSA), the general population can be exposed to BPA in external, internal, and aggregated ways via food, dermal contact (cosmetics and thermal paper), drinking water, swimming, and/or breathing indoor and outdoor air [2]. However, breast milk represents the main vehicle of human intake of BPA, which determines its highest concentrations in the urine of young children [3].

Although BPA is not dangerous in its polymeric form, its transformation in the monomeric form can be realized in acidic or basic solutions and when exposed to UV light. Thus, over time, food and drink containers can become a widespread public health risk [4]. Furthermore, the negative effects of BPA can be evident for children and adolescents, pregnant women, and their embryos, as confirmed by numerous tests on animals in vivo and in vitro [5,6]. Nevertheless, only free (unconjugated) BPA is a weak estrogen [7], and its presence in the different biological matrices is substantially negligible. This is due to an efficient metabolization of BPA together with a biological half-life in humans of less than six hours [8,9].

BPA, as an endocrine disruptor, is able to contribute to or induce several other negative effects, including reproductive, perinatal, and pediatric outcomes, hepatic tumors, lung inflammation, Parkinson disease, abnormal behavior, obesity, diabetes, and reproductive abnormalities in offspring [10]. Furthermore, BPA is able to induce an increase in oxidative stress [11–13].

Usually, BPA is detectable in urine, blood, breast milk, semen, cord blood, fetal serum, placental tissue, and animal fat [14,15], but in urine, its detection frequency is about 75–90% [16,17]. Glucuronic acid of BPA (GlcA–BPA) is also an urinary metabolite of BPA, and it is currently considered the major residue of BPA, both in vitro and in vivo [18], which makes it suitable for molecular epidemiology studies. BPA contributes to lipid peroxidation (LPO), and therefore, as mentioned earlier, to the induction of oxidative stress (OS), which is a biological imbalance that occurs when endogenous and/or exogenous oxidants overtake the level of antioxidant defenses [19–21].

The urinary BPA in children is significantly more concentrated than in adults because they eat, drink, and breathe in greater quantities per kilogram of body weight [15,22]. Furthermore, children are more sensitive and fragile because their metabolism system and organs are not yet fully developed [23]. In particular, infants up to two or three months of age might have higher free-BPA levels in urine since detoxifying enzymes such as UDP-glucuronosyltransferase are not yet fully developed [2,24]. Due to the widespread exposure to BPA and the consequent potential health risk to humans, restrictions and dedicated regulations for the use of this toxic chemical have been suggested worldwide. In 2015, the EFSA [25] reduced the temporary Tolerable Daily Intake (t-TDI) of BPA from 50 to 4 μg/kg bw/day. Consequently, BPA is being replaced with a number of alternatives.

Although the presence of oxidative stress is a known prepatological condition of numerous health effects, including atherosclerosis, cardiovascular disease, cancer, and pregnancy outcomes [26], currently, only a few studies on adults, and very few on children have explored the exposure to BPA in relation to the induction of inflammation, LPO, and OS [27,28]. Thus, the aim of the present study has been to investigate the presence of BPA in the urine of a group of adolescents, its role in the induction of OS, and to confirm the same role of tobacco smoke [29–31]. Furthermore, given that our previous works had shown an unexpectedly decreasing trend in oxidative stress among adolescents, in this work, we wanted to check this contrasting trend again with the other life phases.

To achieve this goal, a sample of urine provided by every one of the 223 young healthy volunteers (7–19 years old) attending three different schools of Chivasso (close to Torino, Piedmont, northwestern Italy) was analyzed to quantify BPA, cotinine, and 15F2t-Isoprostane (15F2t-IsoP). The first was a chemical directly detectable in urine as an internal dose biomarker, the second was a nicotine metabolite to quantify exposure to smoking (an internal dose biomarker, too), and the third was a biomarker of OS. We chose 15F2t-IsoP because it is one of the most stable, sensitive, and non-invasive biomarkers of oxidative stress in urine; this is because it is a specific and stable product of lipid peroxidation that is largely used for in vivo investigations. [32].

2. Materials and Methods

2.1. Selection of Subjects

All the 223 students who voluntarily participated to this study attended three different schools at Chivasso, which is a medium urbanized town with about 27,000 inhabitants (522 inhabitants/km^2)

located at 180 m above sea level close to Torino (the metropolitan city of the Piedmont Region, Italy—890,500 inhabitants). No other selection criteria were adopted to recruit volunteers. Since the subjects were underage, parents and teachers were informed during a public meeting on the objective of this study, and consequently, written informed consent was signed and delivered by each participants' parents. Moreover, the participation of all the subjects took place only after obtaining the assent of the local Ethics committee of "San Luigi" Turin Hospital (session on 11 March 2015 authorization number 27/2015). Samplings were carried out from January to March, involving one class per day, on Wednesday or Thursday, according to a pre-established timetable. A questionnaire was administered, and a urine sample was collected from each student.

2.2. Questionnaire

To each subject, one interviewer administered a questionnaire during school hours. The answers provided information on individual and clinical features, such as age, weight, and height, gender, residence, diet (dinner the day before), hobbies, therapies, and health conditions. The questionnaire used was mainly a synthesis of the most extensive questionnaire "SIDRIA", which has been described in detail elsewhere [33].

2.3. Urine

A spot of urine was collected from each volunteer during the morning sampling to measure the following parameters:

2.3.1. BPA

To exclude contamination from BPA, all the urine samples were collected in BPA-free plastic vessels (polypropylene) and stored at −80 °C until analysis. All the laboratory glass material that was used was washed with methanol and then kept in methanol for 12 hours, which was subsequently analyzed to verify the possible contamination of BPA. Each thawed sample of urine was vortexed, and 700 µL of acetonitrile, 750 µL of ethyl acetate, and 10 µL of BPA-d_{16} (1 ng/µL), which were used as internal standards, were added to each 400-µL urine sample. To facilitate the liquid–liquid extraction (LLE), samples were vortexed for 3 minutes; then, they were centrifuged at 4000 rpm for 15 min, and the supernatants were evaporated to dryness by a gentle stream of nitrogen. The dried extract was dissolved with 125 µL of methanol/water (1:1 *v/v*) and analyzed by HPLC—MS/MS to quantify GlcA–BPA. GlcA–BPA was identified and quantified by liquid chromatography equipped with a low-pH resistant reverse phase column, Kinetex EVO C18 (2.6 µm, 150 × 3.0 mm). The binary solvent system was: (a) acidified ultrapure water with formic acid 0.1% *v/v* and (b) acetonitrile (HPLC ultrapure grade) acidified with formic acid 0.1% *v/v*. The chromatographic separation was carried out at constant flow rate (200 µL/min^{-1}) and constant temperature (23 °C ± 1 °C) by a column thermostat. The solvent linear gradient was from 10% to 30% of B in 5 min, to 65% of B at 30 min, and 95% of B at 33 min. The concentration of solvent B was maintained at 95% for 5 min. The initial mobile phase was re-established for 10 min before the next injection. The injection volume was 20 µL, and quantification was performed by internal standard method (BPA-d_{16}). Quantitative analyses were carried out by tandem mass spectrometry with a 6330 Series Ion Trap LC-MS system equipped with an electrospray ionization source (ESI). The analytes were detected in negative mode. The dry gas (Nitrogen) was at 325 °C, 20.0 psi, and 10 L min^{-1}; capillary voltage was at 2000 V. Data acquisition was made in multiple reaction monitoring (MRM) mode by monitoring the transitions of quasi-molecular ions [M-H]: 227 for BPA, 242 for BPA-d_{16}, 307 for HO_3S–BPA, 403 for GlcA–BPA, and 419 for OH–GlcA–BPA. Procedural blank samples with ultrapure water in the place of urine were collected, extracted, and analyzed by HPLC-MS/MS with the same sample protocol. In the processed blanks, BPA contaminations above the limit of detection (LOD, 0.065 ng·mL^{-1}) were not detected.

2.3.2. Cotinine

Urine samples were prepared for analysis as follows: 10 ml of urine were fortified with 10 µL of cotinine-d_3 as an internal standard, 4 g of NaCl, and 500 µL of NaOH (5 M). Then, 2 mL of $CHCl_3$ was added two times to extract the cotinine by means of LLE for 15 min. Then, each sample was centrifuged for 10 min at 1000× *g*, and the resulting organic phase was collected in a glass tube and evaporated to dryness in a rotary evaporator at room temperature. The dry residue was reconstituted in 200 µL of $CHCl_3$ and transferred into a conical vial for GC-MS determination [34].

2.3.3. 15.F_{2t}-Isoprostane (15F_{2t}-IsoP)

15.F_{2t}-IsoP was measured to quantify OS by the ELISA technique, which was carried out with a specific microplate kit (Oxford, MI, USA) and according to the manufacturer's instructions. To achieve better accuracy in the competitive ELISA method, each sample was diluted 1:4. Our previous paper reports all the details of this procedure [32].

2.3.4. Creatinine

In order to normalize the excretion rate of cotinine, 15F_{2t}-IsoP, GlcA–BPA, and an aliquot of fresh urine were used to quantify the concentration of creatinine (CREA) by the kinetic Jaffè procedure.

2.4. Statistical Analysis

Statistical analysis was performed by means of Stata 12 Statistical Package (Stata Corp LP, Lakeway Drive, TX, USA). Appropriate linear transformation was applied on data whenever suggested by distributional diagnostic plots (symmetry plot, quantile plot) and descriptive statistic inspection (looking at variance stability among categories).

In inspecting the two-way plot of log (ng 15F_{2t}-IsoP/mg CREA) versus log (GlcA–BPA), a non-linear relationship between these variables was detected, suggesting a threshold value of the (GlcA–BPA) on (ng 15F2t-IsoP/mg CREA). So, to estimate a spline function, we used piecewise linear or "hockey stick" robust multiple regression [35] using Box–Cox transformed ng 15F2t-IsoP/mg CREA as the dependent and Box–Cox transformed (GlcA–BPA). This presupposes that two straight lines, with different slopes, and calculating the two slopes and the value of the dependent at which the slope changes (the breakpoint or spline point), can best fit the effect of predictive variables on dependents.

In the model log (ng cotinine/mg CREA), the effects of linear body mass index (BMI), gender, and age classes were also tested and retained in the model as covariates when the 5% significance of the effect was reached or significantly changed the estimates.

3. Results

In Table 1, the characteristics of students enrolled for the study are reported. Numerousness, mean, standard deviation (s.d.), and percentage (%) for gender, age (years), height (m), weight (kg), and smoking exposure (number of cigarettes per day) are shown for the subjects grouped for educational level. Among the 223 students, 18 reported being active smokers (8%), which were all from the 14–19 age group; 52 were passive smokers (23.3%), and 153 were non-smokers (68.7%). In Table 2, cotinine, 15F_{2t}-IsoP, and GlcA–BPA—all expressed as nanograms per 1 milligram of creatinine—are listed according to educational level as mean, standard deviation, minimum, and maximum.

GlcA–BPA shows an increase of concentration proportional with increasing age, even if the intermediate age group (11–14 years) is slightly lower. The same thing is observed also for 15F_{2t}-IsoP and the exposure to tobacco (mainly passively breathed) quantified by cotinine. According to the Box–Cox regression results, the values of the biological markers analyzed were subjected to a logarithmic transformation before carrying out the subsequent analysis. The result of piecewise linear robust regression shows a breakpoint at 1.79 (95% CI: 1.56–2.02; $p < 0.001$) of the effect of

log-GlcA–BPA on log-$15F_{2t}$-IsoP (Figure 1 and Table 3). Thus, the concentration of $15F_{2t}$-IsoP increases exponentially (more than threefold for each one-log unit of GlcA–BPA), when the log-GlcA–BPA concentration overcomes the breakpoint identified at 1.79 log-GlcA–BPA (6 ng/mg CREA). Multiple Linear Regression (MLR) analysis shows a positive effect also of log cotinine concentration on log $15F_{2t}$-IsoP (Table 3). This last effect is evident even considering that a 12% increase of $15F_{2t}$-IsoP is observed for each increment of a log-cotinine unit. Furthermore, the analysis of the relationship between log (ng $15F_{2t}$-IsoP/mg CREA) and age shows a V-shaped trend (Figure 2), with a significant decrease ($p = 0.026$) between infancy (7–10 years old) and the beginning of adolescence (11–15 years old), and then a new increase starting from 15 years of age (Figure 2 and Table 4).

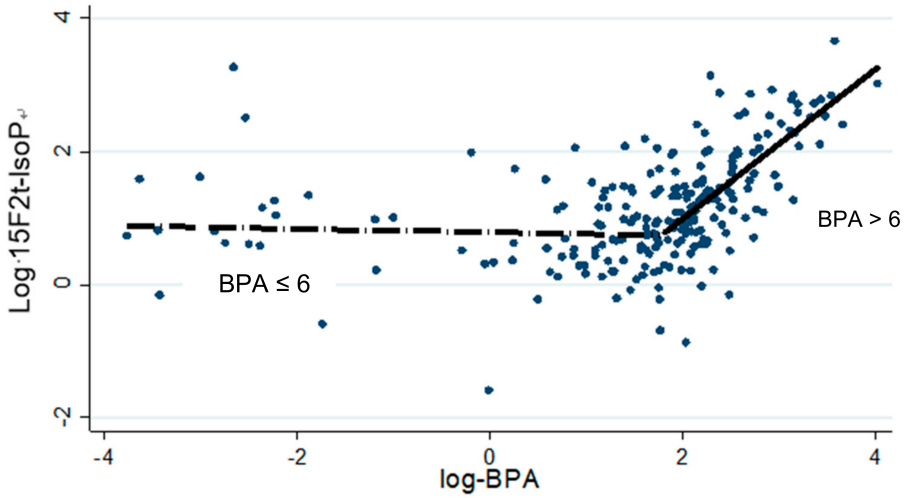

Figure 1. Piecewise linear robust regression of the relation of log glucuronic acid of bisphenol A (GlcA–BPA) on log (ng $15F_{2t}$-IsoP/mg CREA)—(break point at BPA = 6 ng/mg creatinine (CREA), 95% CI: 4.5—7.5). Exp (1.79) = 6.

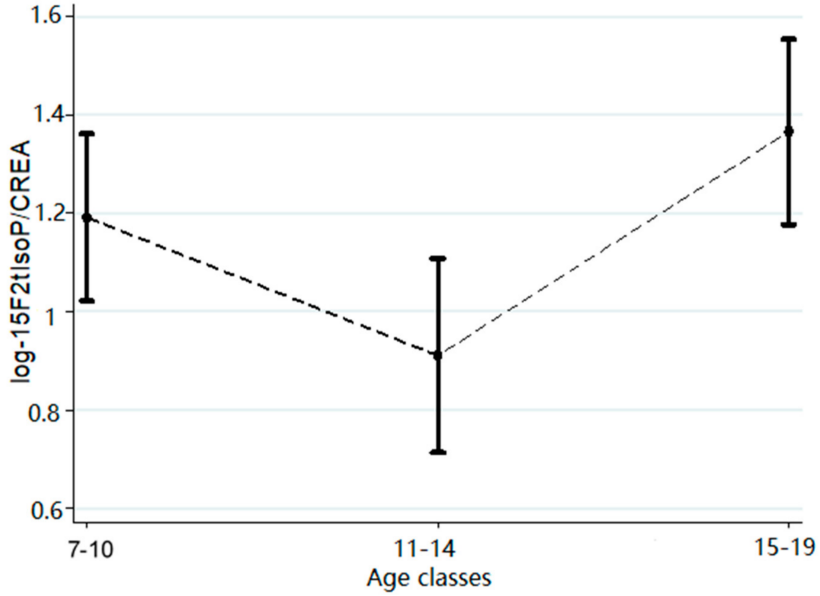

Figure 2. Margins plot of the relation between log 15F2t–IsoP and age classes.

Table 1. Gender, age, height, weight, and number of active and passive smokers in the whole population and in three groups subgrouped according to the three educational level considered.

Characteristics of Students	Primary School (7–10 Years)	Secondary School (11–14 Years)	High School (15–19 Years)	Total
N.	87	34	102	223
Gender N. (%)	Male 47 (54.0%) Female 40	Male 15 (44.1%) Female 19	Male 57 (55.8%) Female 45	Male 119 (53.4%) Female 104
Age (years) Mean ± s.d.	8.87 ± 1.0	11.7 ± 0.8	16.6 ± 1.71	12.8 ± 3.8
Height (m) Mean ± s.d.	1.39 ± 0.08	1.54 ± 0.1	1.71 ± 0.08	1.56 ± 0.17
Weight (kg) Mean ± s.d.	35.6 ± 9.8	45.0 ± 7.5	64.5 ± 12.4	50.2 ± 17.2
Smoking habits N (%)	Active 0 Passive 26 (30%) Not exposed 61 (70%)	Active 0 Passive 5 (14.7%) Not exposed 29 (85.3%)	Active 18 (17.6%) Passive 21 (20.5%) Not exposed 63 (61.9%)	Active 18 (8%) Passive 52 (23.3%) Not exposed 153 (68.7%)

Table 2. Urinary cotinine, 15F2t-IsoP, and total BPA inactivated values in the three groups subgrouped according to the three educational level considered. g-Mean = geometric mean, s.d. = geometric standard deviation, Min = minimum value; Max = maximum value. Units of biological markers are nanograms of every 1 mg of urinary creatinine.

Educational Level	Cotinine [ng/mg CREA]	$15F_{2t}$-IsoP [ng/mg CREA]	Total BPA Inactivated [ng/mg CREA]
	g-Mean (±s.d.) Min–Max	g-Mean (±s.d.) Min–Max	g-Mean (±sd) Min–Max
Primary school (7–10)	11.2 (±8.1) 1.06–382.9	3.3 (±2.2) 0.6–38.8	2.3 (±6.8) 0.02–38.7
Secondary school (11–14)	2.81 (±13.4) 0.1–372.3	2.5 (±2.1) 0.5–17.1	5.4 (±2.5) 0.9–34.4
High school (15–19)	26.3 (±16.8) 0.1–1730.9	3.9 (±2.4) 0.4–23.2	8.4 (±2.2) 0.3–55.4
Total g-mean (±s.d.) min–max	9.8 (±13.9) 0.03–1730	3.2 (±2.8) 0.41–38.8	4.9 (±4.2) 0.02–55.4

Table 3. Pricewise multiple non-linear regression parameters, with means and 95% confidence interval (CI), of log $15F_{2t}$-IsoP as the dependent variable and log (total inactive BPA), log cotinine, and age as predictors.

log $15F_{2t}$-IsoP	Coef.	95% CI Lower limit–Upper Limit		*p*
breakpoint	1.79	1.56	2.02	0.00
breakpoint	1.79	1.56	2.02	0.00
Log (total inactive BPA) < breakpoint	−0.01	−0.10	0.08	0.82
≥ breakpoint	1.11	0.87	1.34	0.00
Log Cotinine (ng/mg CREA)	0.03	0.00	0.06	0.05
<10	0			
Age class 11–14	−0.20	−0.41	0.00	0.05
≥ 15	−0.07	−0.27	0.14	0.53
Constant	0.73	0.59	0.87	0.00

Table 4. Estimated means of log $15F_{2t}$-IsoP by age class adjusted for log (total inactive BPA), log cotinine by means of piecewise non-linear regression.

Age Classes		Means	95%c CI Lower Limit–Upper Limit	$p <$
Age (years old)	<10	1.19	1.02–1.36	NS
	11–14	0.91	0.71–1.11	<0.05
	≥15	1.37	1.37–1.18	NS

4. Discussion

The main objective of this work was to evaluate the environmental diffusion and the possible consequent absorption of BPA in a population of children and adolescents attending primary, secondary, and high school in a city located in Piedmont region, in the northwestern part of Italy. At the same time, we wanted to observe the role of this pollutant in the induction of OS, taking into account as confounders, the role of passive and active exposure to tobacco smoke and age, and other predictors of the same effect. These youth were enrolled as a population that is useful for investigating some environmental conditions as predictors of OS status development as accurately as possible. This is because their life habits lead them to be more in contact with the outside environment and because their lower body weight makes them more sensitive and vulnerable. Regarding this concern, it is also known that young people are still in a phase of development of the body and of their metabolic system, and therefore still fragile and hypersensitive to environmental stimuli.

The OS level was monitored through the quantification of urinary $15F_{2t}$-IsoP concentration, which is a biomarker that is unaffected by diet, potentially confounding the relationship we have investigated [36,37]. Furthermore, the diet was very similar among all the students. This was known from the replies to the questionnaire—they outlined a homogeneous domestic diet—and because they benefit from the same school lunch prepared by the same company according to the requirements imposed by nutritionists working at the local health authority to minimize oxidant food.

Since the exposure to BPA can influence the OS level, urinary GlcA–BPA was measured to understand the role of this contaminant in the onset of $15F_{2t}$-IsoP values. The findings show that the effect of log GlcA–BPA on $15F_{2t}$-IsoP has a threshold value around a breakpoint of 1.79. This suggest that values of GlcA–BPA lower than 4.5 ng/mg of creatinine (exponential value of lower confidential limit) have no measurable effect on isoprostane; conversely, above the breakpoint (6 ng/mg crea), $15F_{2t}$-IsoP grows linearly ($p < 0.005$). To explain this log-linear relationship characterized by a threshold value, we have to remember the higher commitment of the liver to contrast the higher concentrations of this contaminant, or an insufficient sensitivity of analytical technique to detect BPA at lower concentrations. Nevertheless, this last hypothesis seems to be contradicted by the log-linear relationship without the threshold of the $15F_{2t}$-IsoP value versus cotinine. Indeed, the induction of oxidative stress by passive and/or active smoking was confirmed in adolescent subjects independently from age, which was also in our previous paper [38].

The age of the subject proved to be another factor that can significantly influence the $15F_{2t}$-IsoP concentration. In a previous work [38], the $15F_{2t}$-IsoP levels were studied in the 11–15 age group. A slight decrease (6%) was recorded when passing from 11 to 15 years. In the present study, the analysis of $15F_{2t}$-IsoP levels according to age (7–19 years old) highlighted the V-shape previously illustrated. This seems to confirm that the OS experiences a lowering of intensity in the first years considered, and then return to grow regularly. This may result in the establishment and growth of a condition of chronic inflammation until senescence [37,39,40].

Finally, we found that urine GlcA–BPA concentrations were positively but not significantly associated with BMI. Due to its rapid metabolism (half-life less than 6 h), BPA exposure estimates from first morning urine may just represent the exposure at the prior meal (dinner), rather than daily or average exposure level. Given the food indigestion as the main exposure route to BPA, perhaps

more urine samples should be collected throughout the day preceding the sampling to avoid the underestimation of exposure to this contaminant.

We can conclude that the adolescents studied showed an increase in OS dependent from GlcA–BPA higher than 4.8 ng/mg CREA, and from tobacco smoke passively and/or actively breathed. The induction of oxidative stress by GlcA–BPA is a theme that has not yet been analyzed in depth by the International Scientific Community. The public health authorities must consider it in a careful manner and without forgetting the other bisphenols that are now present in the living environment. Thus, the evidence of these risky conditions for public health may represent a platform for designing new preventive strategies addressed at promoting adolescent health in a sensitive period of growth, sexual differentiation, and brain development. Therefore, further studies on new and safer materials that have the least impact on the environment and human health are crucial.

The main results obtained in this work are: GlcA–BPA causes an increase in OS in the adolescents selected for the study, but only starting from 6 ng/mg of CREA. In addition, the passively breathed tobacco smoke is able to induce an increase of the OS. Therefore, the promotion of health must also consist of the preventive contrast to BPA and all the bisphenols still present in the living environment.

5. Limitations and Future Purposes

A limitation of this study is that we planned a cross-sectional study design in different age ranges. Besides, our data had not been collected to specifically assess diet or other potential cofounders, such as environmental pollution. Instead, we intend to plan a longitudinal study to confirm all the trends found in this fist explorative research, both in terms of relationships between oxidative stress and BPA exposure and of possible roles of different confounding factors.

6. Conclusions

Apart from the already demonstrated role of passive exposure to tobacco smoke [41], an increase of oxidative stress was observed also consequently to exposure to BPA, but only from 6 ng/mg of CREA upwards. In effect, 15F2t-isoprostane has proved to be positively correlated with exposure to BPA and tobacco smoke. This highlights the role of the risk factor of these pollutants in the increase of oxidative stress. Thus, the prevention and contrast regarding the exposure to BPA and passive tobacco smoke represent an important tool to promote the highest health standard in a category of subjects that is so particularly sensitive to the quality of the living environment.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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Tobacco Smoke Exposure, Urban and Environmental Factors as Respiratory Disease Predictors in Italian Adolescents

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Abstract: Risk monitoring in childhood is useful to estimate harmful health effects at later stages of life. Thus, here we have assessed the effects of tobacco smoke exposure and environmental pollution on the respiratory health of Italian children and adolescents using spirometry and the forced oscillation technique (FOT). For this purpose, we recruited 188 students aged 6–19 years living in Chivasso, Italy, and collected from them the following data: (1) one filled out questionnaire; (2) two respiratory measurements (i.e., spirometry and FOT); and (3) two urine tests for Cotinine (Cot) and 15-F_{2t}-Isoprostane (15-F_{2t}-IsoP) levels. We found a V-shape distribution for both Cotinine and 15-F_{2t}-IsoP values, according to age groups, as well as a direct correlation ($p = 0.000$) between Cotinine and tobacco smoke exposure. These models demonstrate that tobacco smoke exposure, traffic, and the living environment play a fundamental role in the modulation of asthma-like symptoms ($p = 0.020$) and respiratory function ($p = 0.007$). Furthermore, the results from the 11–15-year group indicate that the growth process is a protective factor against the risk of respiratory disease later in life. Lastly, the FOT findings highlight the detrimental effects of tobacco smoke exposure and urbanization and traffic on respiratory health and asthma-like symptoms, respectively. Overall, monitoring environmental and behavioral factors in childhood can provide valuable information for preventing respiratory diseases in adulthood.

Keywords: environmental pollution; tobacco smoke exposure; childhood; spirometry; forced oscillation technique

1. Introduction

Childhood is a crucial stage of life during which harmful lifestyle habits can be easily and unconsciously acquired. This can lead to changes in metabolic pathways and unfavorable health effects in adulthood [1,2]. For example, monitoring hazard conditions and lifestyle habits during growth has been shown to be a useful parameter to estimate different risky conditions later in life, such as

cardiometabolic diseases and obesity [3]. Furthermore, deleterious lifestyle habits are often worsened by exposure to environmental pollution and tobacco smoke, factors to which children and young adults are commonly exposed worldwide [4,5].

While adults avail themselves of different strategies to counteract these risk factors, children who live, play, and study in urbanized areas and/or are in close contact with smokers often become forcibly exposed. Moreover, when, for some reason, the immune system becomes partially compromised, some clinical manifestations such as lower respiratory tract infection, ear infection, and lifelong cardiovascular risk are further worsened [6]. The respiratory system is considered a primary target of air pollution. Since children breathe a proportionately greater volume of air than adults and they have smaller caliber airways, they are more likely to develop inflammation-related illnesses and increase their overall level of oxidative stress [7–9]. In addition, as children tend to spend more time outdoors, they are more likely to experience small airway obstruction in the presence of high levels of air pollution [10]. The respiratory health of children can also be compromised by passive and active exposure to tobacco smoke. In fact, tobacco smoke is still the main preventable risk factor for respiratory, allergic, and cardiovascular disease, as well as for cancer [11]. At earlier stages of life, passive exposure to smoke or the beginning of active addiction to tobacco can be particularly damaging. This can be ascribed to the fact that individuals are more vulnerable to the harmful effects of tobacco during early stages of growth. In this regard, mounting evidence suggests that tobacco smoke exposure in early puberty may have a negative impact on health across generations [12,13]. This is particularly important in Italy, where over 20% of 15-year-old adolescents smoke at least once a week and more than 13% smoke every day [11,14–16].

In this scenario, we aimed to assess behavior towards some of the most widespread and harmful determinants of lifestyle habits and respiratory health, such as passive and active tobacco smoke and environmental pollution in relation to age in a large population of children and adolescents in Italy. Finally, we sought to identify possible alterations in respiratory functions using spirometry and the Forced Oscillation Technique (FOT). This can allow the identification of differences between the two breathing measurement techniques, among a different range of ages, and between different environmental conditions, including exposure to tobacco smoke.

2. Materials and Methods

2.1. Study Participants

For the study, 204 students were recruited from elementary, middle, and high schools of Chivasso, a small urbanized town close to Turin, Italy. Chivasso is inhabited by 26,976 people (on 1 January 2018, according to the Italian Institute of Statistics), and it extends over 51.24 km^2, with a density of 526.48 inhabitants/km^2. To recruit the subjects, the following criteria were adopted: a) living in or nearby Chivasso and b) being aged between 6 and 10 years (elementary schools), 11 and 15 years (middle schools), and 15 and 19 years (high schools). Because the subjects were underage, parents or guardians of children were asked to sign an informed consent for study enrollment according to the Helsinki Declaration. Sampling was carried out from January to March 2016 according to a pre-established timetable. At the end of the samplings, after the evaluation of the respiratory results, 188 subjects were selected. For each subject, we collected the following data:

1. Questionnaire: Questions selected from the most extensive SIDRIA questionnaire [16], as described previously [17], were administered to each subject enrolled. This information was used to establish individual and clinical features (i.e., age, weight, height, Body Mass Index –BMI-, gender, residence, hobbies, therapies, and health conditions). Questions on tobacco smoke and urbanization included a parental and subjective evaluation of the exposure (absent, low, moderate, or high). The questionnaire was also structured to gain in-depth knowledge of personal lifestyle habits of the subjects and to gather information on the main asthma-like symptoms, such as asthma attacks, wheeze with breathlessness, current use of treatments for asthma, current hay

fever/nasal allergies, waking with chest tightness, being woken by shortness of breath, and being woken by coughing [18].

2. Spirometry measurements: These were expressed as maximal expiratory flow–volume curves to establish forced vital capacity (FVC), forced expiratory volume in the first second (FEV_1), maximal expiratory flows at peak 50%, 25%, and among 25–75% of FVC (PEF, FEF_{50}, FEF_{25}, FEF_{25-75}) and the FEV1/FVC ratio. The instrument (CPFS/D, MGC Diagnostics Corporation, St Paul, MN, USA) was calibrated daily with a 3 L syringe. After a brief training, the measurements were carried out in accordance with the current ATS/ERS standards [19] and repeated until the volume variability did not exceed 150 mL for at least 2 times in order to comply with both within- and between-maneuver criteria [20].
3. Respiratory mechanics: These were measured by FOT by means of a Resmon Pro FULL device (Restech, Milan, Italy). This method is noninvasive and employs small-amplitude pressure oscillations superimposed on the normal breathing, not requiring the performance of respiratory maneuvers [21]. A couple of measurements of at least 10 breaths each were obtained from each individual. The quality of breath was assessed through a specific algorithm contained inside the device and subsequent mathematical evaluation. Resistance and reactance obtained at a frequency of 5 Hz were used for the analysis.
4. Morning urine spot: This test was performed to measure the following parameters:

    I. Cotinine. Cotinine measurements were carried out to objectively quantify the passive and active exposure to tobacco smoke. Cotinine levels were also regarded as a possible inductor of oxidative stress (OS) imbalance [22,23]. Urine samples were prepared for analysis as previously described [10,20,24]. Gas chromatography mass spectrometry (GC-MS) analysis was performed using an Agilent Technologies 6890 GC, interfaced to a 5973 MSD Inert Agilent mass spectrometer. The MS operated in electron impact and SIM mode. The limit of detection (LOD) and limit of quantification (LOQ) were 0.01 μg mL^{-1} and 0.02 μg mL^{-1}, respectively. The coefficient of variation (CV), calculated to test repeatability, was below 5% for both Cotinine and the internal standard;

    II. 15-F_{2t}-Isoprostane (15-F_{2t}-IsoP). 15-F_{2t}-IsoP was measured to quantify OS by the ELISA technique using a specific microplate kit (Oxford, MI, USA) according to the manufacturer's instructions. To achieve better accuracy in the competitive ELISA method, each sample was diluted 1:4. The procedure is described in more detail elsewhere [25,26]; and

    III. Creatinine (Crea). Crea quantification was performed by the kinetic Jaffè procedure in order to normalize the excretion rate of Cotinine and 15-F2t-IsoP [20].

5. Statistical analyses: They were all carried out using the Stata 14 Statistical Package (Stata Corp LP, Lakeway Drive, TX, USA). In univariate analysis, the variables in ordinal or interval scale were compared between gender and age classes through the non-parametric Kolmogorov–Smirnov 2 sample equality-of-distributions test and the Kruskal–Wallis equality-of-populations rank test. The frequency differences were tested with Pearson's chi-squared test. Differences with a $p < 0.05$ were considered significant. To analyze the determinants of 15-F_{2t}-IsoP, multiple linear regression analysis was performed using Box–Cox-transformed [27] 15-F_{2t}-IsoP as the dependent variable. Height, age (6–10, 10–15, or >15-year groups), log Cotinine, and smoking exposure (recorded as yes or no) were used as predictive variables. In all models, variables were retained when they reached a level of 5% significance. To assess the effect of covariates on lung function parameters (measured through spirometry and FOT), we compared the findings of the spirometric parameters with the Global Lung Function Initiative (GLI) reference values [28], assuming as cut-off of "normal" values the lower 10% confidence limit of normality (LLN), as recommended by GLI authors. A sub-sample of asymptomatic subjects not exposed to tobacco smoke was selected from the whole group to calculate the reference values for FOT still missing in a well-stabilized form. This was achieved through multiple regression analysis of Box–Cox-transformed resistance

and reactance calculated at a frequency of 5 Hz as dependent variables, selecting age, height, weight, and gender (female as reference value) as independent variables. The limits of normal test variability were computed following GLI recommendations. For spirometric values, the normal values were defined by comparing them with the lower limits of normality (LLN), while FOT and oscillatory resistances were compared with elastance and the upper limits of normality (ULN). A set of multiple logistic regression analyses were performed using the abnormality of findings as dependent variable, smoking, and traffic exposure as predictors, and age, gender, and BMI as confounders. A p value ≤ 0.05 (two-tailed) was considered significant in all tests. All variables that were not significant at the 5% level and not influencing other parameters were excluded.

2.2. Compliance with Ethical Standards

All procedures performed in this study involving human participants were in accordance with the ethical standards of the local Ethics Committee of "*San Luigi Gonzaga*" Hospital (session on 11 March 2015, number 27/2015) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

3. Results

The study was circumscribed to 188 collaborative subjects who had provided valid results with respect to the respiratory parameters analyzed. Table 1 shows the main anthropometric characteristics of the subjects according to gender (top) or age group (6–10, 11–15, and >15 years) (bottom), in order to highlight whether there were differences in respiratory variables [18,29] and in OS levels [30–32], principally because the population was almost heterogeneous in terms of development stages but also in terms of age.

Table 1. Anthropometric characteristics of the subjects according to gender (**top**) or age group (**bottom**).

Individual Characteristics		Total (n = 188)	Male (n = 103)	Female (n = 85)	p Value (KS/KW test)
Age (years) Mean ± S.D.		12.9 ± 3.8	12.9 + 3.9	12.9 + 3.6	0.719
Height (m) Mean ± S.D.		1.6 ± 1.7	1.6 + 1.9	1.5 + 1.3	0.729
Weight (Kg) Mean ± S.D.		50.1 ± 17.3	52.7 + 19	46.8 + 13.8	0.090
BMI Mean ± S.D.		19.6 ± 3.8	19.9 + 4	19.1 + 3.4	0.229
BMI IOTF No. (%)	Underweight	17 (9%)	7 (6.8%)	10 (11.6%)	
	Normal weight	132 (69.8%)	76 (73.8%)	56 (65.1%)	
	Overweight	27 (14.3%)	10 (9.7%)	17 (19.8%)	
	Obese	12 (6.9%)	10 (9.7%)	2 (3.5%)	
Smoking habits No. (%)	No	134 (70.9%)	71 (68.9%)	63 (73.2%)	
	Passive	41 (21.7%)	20 (19.4%)	21 (24.4%)	
	Active	14 (7.4%)	9 (11.7%)	5 (5.8%)	
Isoprostane (ng/mg Crea) Mean ± S.D. (Min–Max)		4.5 ± 4.7 (0.2–38.8)	4 ± 3.8 (0.8–17.7)	5.1 ± 5.7 (0.2–38.8)	0.06
Cotinine (ng/mg Crea) Mean ± S.D. (Min–Max)		102 ± 196.9 (0.1–1730.9)	92.6 ± 151.4 (0.1–742.5)	115.5 ± 241 (0.1–1730.9)	0.15
FVC Mean ± S.D.		3.5 ± 1.5	3.8 ± 1.7	3.1 ± 1.2	**0.00**
FEV1 Mean ± S.D.		3.1 ±1.3	3.4 ± 1.5	2.7 ± 0.9	**0.00**
FEF25 Mean ± S.D.		5.4 ± 2.3	6 ± 2.7	4.8 ± 1.4	**0.00**
FEF50 Mean ± S.D.		3.9 ± 1.7	4.3 ± 2.1	3.6 ± 1.1	**0.00**
FEF25–75 Mean ± S.D.		3.5 ± 1.6	3.9 ±1.7	3.2 ± 1.1	**0.00**
FEV1/FVC Mean ± S.D.		0.8 ± 0.1	0.9 ± 0.04	0.8 ± 0.08	**0.011**
R5 tot Mean ± S.D.		4.2 ± 1.7	4.2 ± 1.9	4.2 ± 1.4	**0.01**

Table 1. *Cont.*

	6–10 years old (n = 74)	11–15 years old (n = 53)	15 + years old (n = 61)	p Value (KS/KW test)
Isoprostane (ng/mg Crea) Mean ± S.D.	4.7 ± 5.3	3.8 ± 4.2	5.1 ± 4.5	**0.00**
Cotinine (ng/mg Crea) Mean ± S.D.	74.6 ± 109.7	33.2 ± 111.6	196.7 ± 284.7	**0.00**
FVC Mean ± S.D.	2.2 ± 0.4	3.7 ± 1.3	4.9 ± 1.3	**0.00**
FEV1 Mean ± S.D.	1.9 ± 0.3	3.3 ± 1	4.3 ± 1.2	**0.00**
FEF25 Mean ± S.D.	3.7 ± 0.6	5.7 ± 1.7	7.4 ± 2.4	**0.00**
FEF50 Mean ± S.D.	2.7 ± 0.5	4.1 ± 1.2	5.4 ± 1.9	**0.00**
FEF25–75 Mean ± S.D.	2.4 ± 0.5	3.7 ± 1.1	4.9 ± 1.8	**0.00**
FEV1/FVC Mean ± S.D.	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	**0.00**
R5 tot Mean ± S.D.	5.7 ± 1.3	4 ± 1.3	2.8 ± 0.7	**0.00**
X5 tot Mean ± S.D.	−1.8 ± 0.8	−1.2 ± 0.7	0.9 ± 0.3	0.54

BMI = Body Mass Index; IOTF = International Obesity Task Force; FVC= Forced Vital Capacity; FEV1 = Forced expiratory Volume in the First Second; FEF= Maximal Expiratory Flows; FEV1/FVC = FEV1/FVC ratio; Crea Creatinine; R5 tot= total resistance; X5tot = total reactance

The Kruskal–Wallis test showed no significant differences between genders for the anthropometric variables tested, whereas it detected significant differences for biologic and lung-function markers for all variables among the different age groups (Table 1). The BMI, categorized according to the IOTF criteria, indicates that over 70% of subjects had a normal BMI, while 21% were overweight or obese. A V-shape trend (Table 1, second part) was found in oxidative stress levels according to age groups (decrease in the 11–15 group, p = 0.00) while no significant differences were found for sex. Among the 188 students, 14 reported being active smokers (7.4%), 41 passive smokers (21.7%), and 134 non-smokers (70.9%). Table 1 also reports the means and standard deviations (SDs) of the following lung function parameters: FVC, FEV_1, maximal expiratory flows at peak 50%, 25%, 25–75% of FVC, and inspiratory, expiratory and total resistance, as measured by FOT at 5 Hz of frequency. All these parameters proved to be homogeneous in the three age groups (KS test = NS). Alike, no significant differences in respiratory parameters were found between males and females. Finally, Table 1 shows the means and SDs of 15-F_{2t}-IsoP and Cotinine, expressed as ng/mg of Crea according to gender and age group.

Multiple non-linear regression performed (Table 2) on the entire population shows that the log Cotinine values, stratified and adjusted for age group, oxidative stress levels and BMI, display a V-shape trend (Figure 1).

In particular, the analysis shows a direct and linear regression (p = 0.000) between Cotinine and exposure to tobacco smoke, with an increase of 13% and 15% for passive and active exposure, respectively (passive B = 1.17, CI = 95% (1.01–1.34); active B = 1.19, CI = 95% (0.97–1.41)) if compared to non-smokers.

Moreover, the V-shape trend is maintained even with the stratification for tobacco smoke exposure, with an overall 12% decrease in the >15 age group (p = 0.050) in comparison with the other age groups.

The study participants were also asked to provide answers to questions selected from the SIDRIA questionnaire [16], as described previously [17], with the aim to establish potential relationships between respiratory health, quantified through respiratory flow parameters (dependent variable), and symptoms and some risk factors such as tobacco smoke exposure, traffic, and living environment (independent variables). Using normal limits, 15 subjects had non-normal total resistance at 5 Hz values (R5-tot) (7.9%). In particular, Figure 2 illustrates the logistic regression analysis results obtained using asthma-like symptoms as the dependent variable and tobacco smoke exposure, traffic, and living environment as independent variables, adjusted for age and gender.

Table 2. Multiple non-linear regression parameters.

Independent Variables		Predictive Margins (95% C.I.)	*p*
Total sample	No	1.04 (0.72–1.36)	**0.000**
	Passive	1.17 (1.01–1.34)	
	Active	1.19 (0.97–1.41)	
Nosmokers	6–10 years old	1.5 (1–2)	**0.050**
	11–15 years old	0.3 (−0.2–0.8)	
	15 + years old	1.5 (0.9–2.1)	
Passive smokers	6–10 years old	4.8 (4.2–5.5)	
	11–15 years old	3.6 (2.8–4.4)	
	15 + years old	4.8 (4.1–5.5)	
Activesmokers	6–10 years old	5.1 (3.9–6.2)	
	11–15 years old	3.8 (2.6–5)	
	15 + years old	5.0 (4.1–6)	

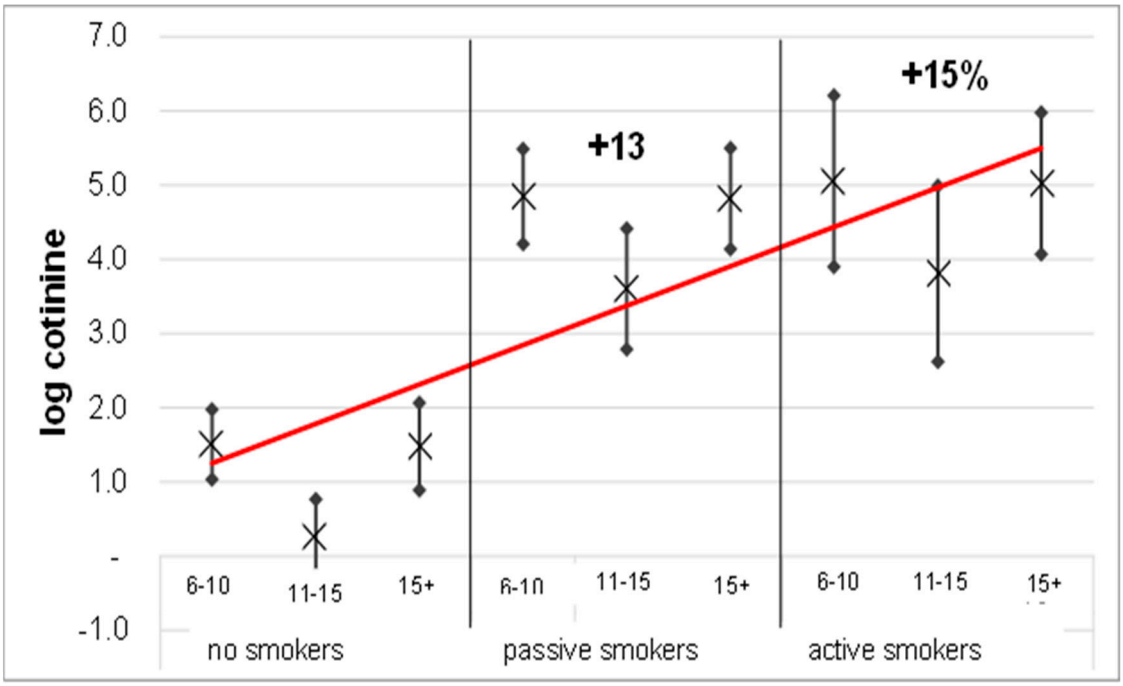

Figure 1. Multiple non-linear regression between log Cotinine (dependent variable) and tobacco smoke exposure, adjusted and stratified for the three age groups. For each groups the figure reported the mean (×), the predictive margins (•) and the regression between log cotinine and tobacco smoke exposure (red line).

Furthermore, Figure 3 shows the logistic model with the FEV_1/FVC ratio (on the left) and FOT R5-tot (on the right) as dependent variables, and tobacco smoke exposure, traffic, and living environment as independent variables, adjusted by age and gender. Both models clearly show that tobacco smoke exposure, traffic, and living environment play a role in the modulation of asthma-like symptoms and respiratory function.

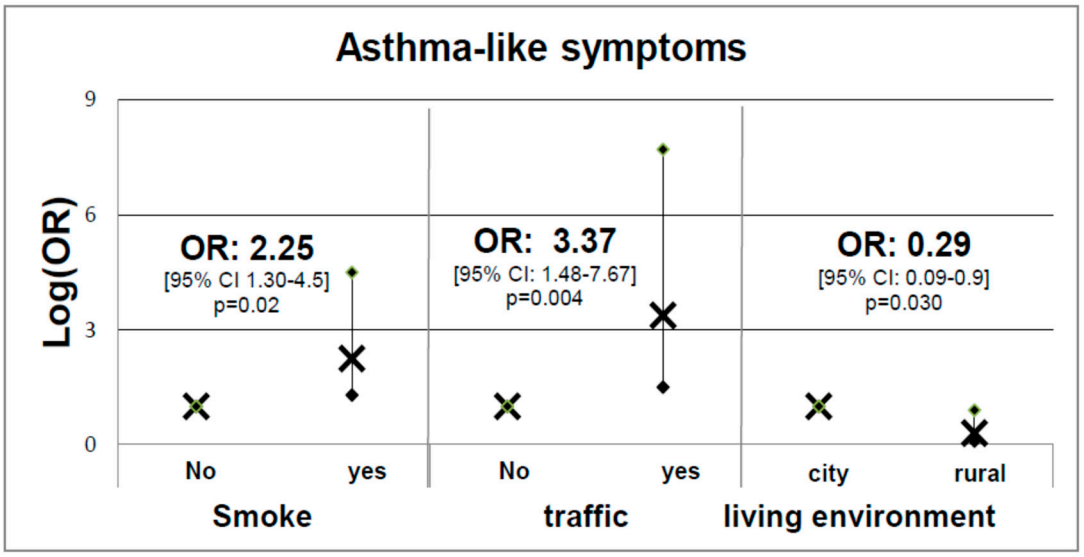

Figure 2. Logistic regression analysis using asthma-like symptoms as the dependent variable and tobacco smoke exposure, traffic, and living environment as independent variables, adjusted for age and gender. For each groups the figure reported the mean (×) and the predictive margins (•).

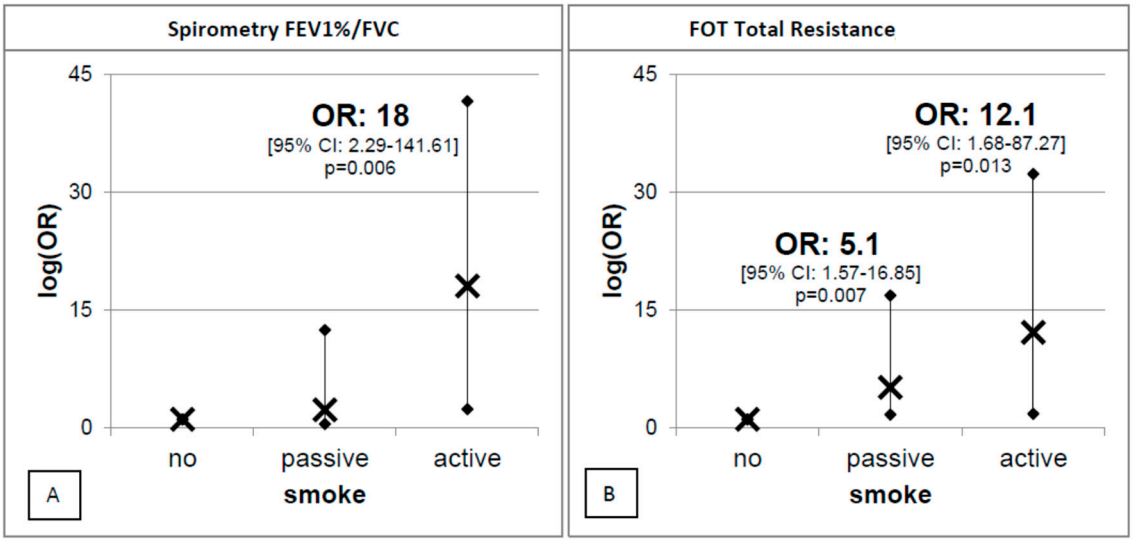

Figure 3. Logistic models with the FEV1/FVC ratio (**A**) and the forced oscillation technique (FOT) total resistance obtained at a frequency of 5 Hz (**B**) as the dependent variable, and tobacco smoke exposure, traffic, and living environment as independent variables, adjusted for age and gender. For each groups the figure reported the mean (×) and the predictive margins (•).

With regard to the first model depicted in Figure 2, asthma-like symptoms were more frequently found in children exposed to tobacco smoke (OR = 2.25 (95% CI: 1.30–4.50); p = 0.020) or living in high-traffic areas (OR = 3.37 (95% CI: 2.29–141.61); p = 0.004). In contrast, living in rural areas seems to have a moderate protective role in terms of respiratory health in the subjects considered (OR = 0.29 (95% CI: 0.09–0.90); p = 0.030). The negative role of tobacco smoke is also confirmed in the second GLM model (Figure 3), which shows how FEV_1/FVC and FOT parameters are negatively influenced upon tobacco smoke exposure as this latter increases substantially the FEV_1/FVC ratio (OR = 18 (95% CI: 2.29–141.61); p = 0.006) but only in active smokers. Furthermore, Figure 3B shows how tobacco smoke exposure worsen significantly FOT R5-tot (OR = 5.1 (95% CI: 1.57–16.85); p = 0.007) and active smokers (OR = 16.8 (95% CI: 1.68–87.27); p = 0.013) (Figure 3B), indicating a better sensitivity of FOT with

regard to early damage to respiratory pathways. No significant relationships were detected between non-normal FVC and FEV_1 and smoke exposure, traffic, and living environment.

4. Discussion

In this study, we aimed to investigate some aspects of youth health through the analysis of specific determinants of health risk factors such as age, tobacco smoke exposure, and air pollution. To this end, we evaluated the respiratory and health status in a group of Italian children and adolescents using age, tobacco smoke exposure, environmental living, oxidative stress, and body composition (BMI) as independent variables. We also explored respiratory health, measured objectively by spirometry and FOT and subjectively by a questionnaire, using passive and active tobacco smoke, intensity of automotive traffic, and urbanization level as independent variables.

Oxidative stress concentrations and BMI levels were found to be closely related to age groups, as already found in our previous works [20,33], but in the deepened analyses we did not find statistical correlations with lung parameters and the other variables.

Figure 1 display an evident V-shape distribution of log Cotinine values sub-grouped according to the three age groups, which shows a substantial decrease in the intermediate age group. In particular, the findings relative to the intermediate age group (11–15 years) underscore the importance of the role played by the growth process in the respiratory health of a mature individual. This particular role in growth-period processes is indeed characterized by a particular hormonal change triggering the activation of enzyme systems, and this seems to give an acceleration of enzyme systems and to protective metabolism. This, in turn, enhances the hormonal and metabolic action, likely lowering the concentration of the analytes measured in this study and yet to be investigated with future longitudinal studies [34,35].

With regard to the effects induced by exposure to tobacco smoke we show a direct correlation between Cotinine load and tobacco smoke exposure in the three age groups (Figure 1). Furthermore, we show that tobacco smoke exposure is not only an inductor of asthma-like symptoms (Figure 2, left-hand side) but also a factor that increases both the FEV1/FVC ratio (Figure 3A) and the total resistance measured by FOT (Figure 3B). In particular, the harmful effect of smoke on respiratory functions is clearly evidenced by both the spirometry and FOT results. Overall, these two techniques appear to be extremely useful and reliable for the assessment of respiratory function in school-age subjects, showing a reduction in respiratory function in active smokers, which is particularly alarming considering the young age of the subjects involved in the study. On the other hand, the impact of secondhand smoke on functional parameters is only revealed by FOT, which appears to be a more sensitive technique than spirometry in detecting small differences between non-smokers and passive smokers. Moreover, FOT seems to be more easily tolerated by children than spirometry, and thus much easier to perform, in good agreement with a previous report [36]. FOT also appears to be more sensitive than spirometry in early detection of increased airway resistance and, more generally, of precocious damage to the respiratory system, making this technique particularly suited to screen young populations.

5. Conclusions

Exposure to traffic and urban residences are important determinants of asthma-like symptoms, which can also be induced by a number of other independent risk factors. Altogether, our findings indicate that monitoring childhood growth trajectories can provide us with important information that can be used to conceive and structure early interventions aimed at preventing the development of health risk in adolescents, a strategy that has already proven successful in reducing the incidence of age-related chronic illnesses such as respiratory or cardiovascular disease and obesity [37].

A second and perhaps more important lesson is that a thorough analysis of tobacco smoke exposure at young ages is clearly needed to prevent the harmful effects of urban environmental pollution on respiratory health [38]. In addition, active and passive exposure to tobacco smoke, to which young

people are particularly sensitive, is another important inductor of respiratory disease that needs to be taken into account, especially in view of the fact that in adolescents the onset of smoking habit is still growing [11].

One of the strengths of this study is the number of subjects involved and the wide age range of the study participants: 188 subjects, from 7 to 19 years old. A limitation of the study is the qualitative estimation of exposure to motor vehicle traffic and the type of urban or rural residence, which was based on subjective responses to the questionnaire, and the lack of information on allergies.

Overall, passive and active tobacco smoke exposure in adolescents must be strongly reduced through a powerful educational action aimed at spreading awareness, in preventive terms, and with the utmost clarity on various smoke-related risks such as respiratory and cardiovascular diseases. Furthermore, we have to stress the need for policies aimed to counteract the influence of smoking parents and grandparents on adolescents [39].

Finally, from a clinical and functional standpoint, our study underscores that even exposure to urban environmental pollution can affect respiratory health since childhood. Thus, strategies for reducing air pollution in heavily urbanized areas should improve health outcomes while reducing heath care expenditures.

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PLOS ONE

RESEARCH ARTICLE

Physical activity and lung function—Cause or consequence?

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Data Availability Statement: The data used in this analysis contain sensitive and identifying personal information from participants in multiple centers across Europe. The participants of the ECRHS study did not provide consent that their data be made public and permission to do so has not been granted by all relevant center-based ethical committees. ECRHS has a data sharing policy and will make the data available upon request to qualified researchers working within institutions with evidence that they comply with current GDPR ethical and professional standards and requirements if all local participating centers are

Abstract

Concerns exist that the positive association of physical activity with better lung function, which has been suggested in previous longitudinal studies in smokers, is due to reverse causation. To investigate this, we applied structural equation modeling (SEM), an exploratory approach, and marginal structural modeling (MSM), an approach from the causal inference framework that corrects for reverse causation and time-dependent confounding and estimates causal effects, on data from participants in the European Community Respiratory Health Survey (ECRHS, a multicentre European cohort study initiated in 1991–1993 with ECRHS I, and with two follow-ups: ECRHS II in 1999–2003, and ECRHS III in 2010–2014). 753 subjects who reported current smoking at ECRHS II, with repeated data on lung function at ECRHS I, II and III, physical activity at ECRHS II and III, and potential confounders at ECRHS I and II, were included in the analyses. SEM showed positive associations between physical activity and lung function in both directions. MSM suggested a protective *causal* effect of physical activity on lung function (overall difference in mean β (95% CI), comparing active versus non-active individuals: 58 mL (21–95) for forced expiratory volume in one second and 83 mL (36–130) for forced vital capacity). Our results suggest bi-directional

able to gain relevant permissions (contact via ECRHS data manager (James Potts, j.potts@imperial.ac.uk) and program manager (Sabrina Kapur, sabrina.kapur@imperial.ac.uk); Professor Debbie Jarvis, d.jarvis@imperial.ac.uk, or any member of the ECRHS Steering Committee, www.ecrhs.org/steering.htm, i.e. Professor Cecilie Svanes, cecilie.svanes@uib.no).

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causation and support a true protective effect of physical activity on lung function in smokers, after accounting for reverse causation and time-dependent confounding.

Background

Previous longitudinal population-based studies suggest a protective effect of physical activity on lung function levels among active smokers [1,2]. However, the potential for reverse causation remains a common criticism, even in longitudinal studies, as both lung function and physical activity vary over time, and previous lung function levels may have affected baseline physical activity levels. This is further complicated by the possibility of time-dependent confounding, which is where a time-varying confounder (e.g., weight) is affected by previous levels of the exposure (i.e. physical activity). One study reported that the role of time-dependent confounding in the association between physical activity and lung function was of negligible magnitude, but did not consider the influence of diet, which is closely related to physical activity and weight [3]. We investigated the potential role of reverse causation and time-dependent confounding on the association between physical activity and lung function among active smokers using repeated data from the European Community Respiratory Health Survey (ECRHS). We used statistical techniques that, unlike standard statistical methods, provide unbiased results in the presence of time-dependent confounding: structural equation modeling (SEM)–an exploratory approach and marginal structural modeling (MSM)–a causal approach.

Methods

Study population

The ECRHS multicentre cohort study collected repeated detailed information on environmental, lifestyle and respiratory health factors from adults, who were sampled in 30 centres (located in 13 European countries and Australia) and were evaluated in 1991–1993 (ECRHS I), 1999–2003 (ECRHS II) and 2010–2014 (ECRHS III). Details of the study design have already been published [4,5]. For this analysis, from the 1,578 subjects who had reported current smoking at baseline (i.e. ECRHS II in our analyses), we excluded the 488 subjects without lung function data at all ECRHS assessments, 62 subjects without physical activity data at both ECRHS II and III, and 275 subjects without dietary data at either ECRHS II or III. A total of 753 subjects from 18 centres were included in our study population (a flow-chart is provided S1 Fig in online S1 File).

Lung function

Pre-bronchodilation forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were measured at each survey according to American Thoracic Society recommendations [6].

Physical activity

At ECRHS II and III, information of usual vigorous physical activity frequency (never, $\leq$once a month, once a week, 2–3 times per week and $\geq$4 times per week) and duration per week (none, 30 minutes per week, 1 hour per week, 2–3 hours per week and $\geq$4 hours per week) was obtained using interviewer-administered questionnaires, at the same time as when lung function was measured. Participants were classified as either physically active if they had

reported ≥2 times and ≥1 hour per week of vigorous physical activity, or non-active otherwise [2]. This "active" variable thus represents a combination of physical activity frequency and duration, and it has been shown to be associated with FEV_1 and FVC in smokers from the ECRHS [2].

Other relevant information

Data on sociodemographic and clinical variables, and other lung function risk factors, were collected using questionnaires: sex, age at baseline (i.e. ECRHS II), age completed full-time education (<17 years; 17–20 years; >20 years), occupation (management/professional/non-manual; technical/professional/non-manual; other non-manual; skilled manual; semiskilled/unskilled manual; other/unknown), childhood respiratory infection (yes/no) and occupational exposure to biological dust, gas/fumes or pesticides (yes/no). Number of pack-years smoked (calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked), second-hand smoke exposure (yes/no) and menopausal status in women (pre-menopausal/post-menopausal) were assessed at each survey. Dietary habits were collected by food frequency questionnaire once, for two centres at ECRHS II and 16 centres at ECRHS III, enabling the derivation of the alternative healthy eating index (AHEI-2010—a continuous measure of diet quality that is based on foods and nutrients predictive of chronic disease risk, range 0–110) [7] at either time-point. Height and weight (and hence body mass index (BMI)) were measured at each survey.

Statistical analyses

Fig 1 depicts the hypothetical causal relationships tested in this study. Because physical activity was only assessed at ECRHS II and III, we considered, for both *t* = ECRHS II and III, the cross-sectional association between usual physical activity (i.e. the assessment of average

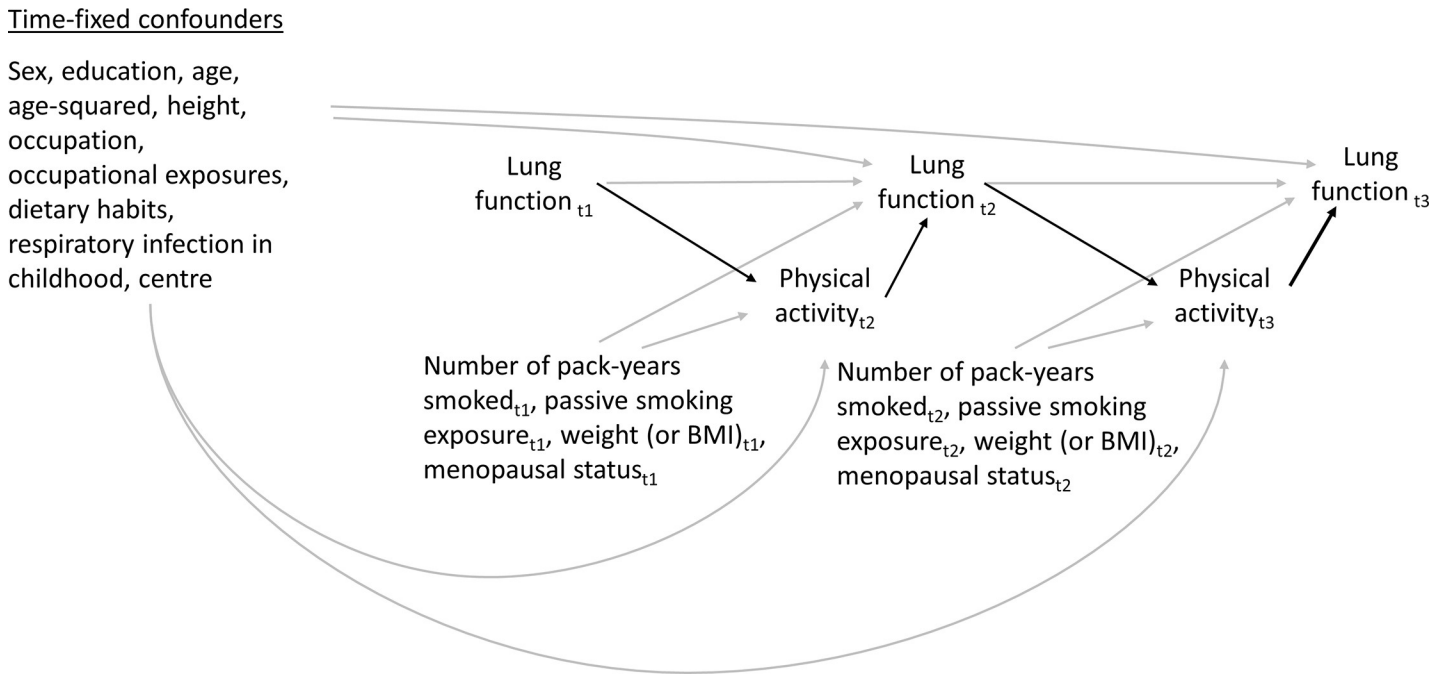

Fig 1. Directed acyclic graph showing potential time-fixed and time-dependent confounders of the association between physical activity and lung function over time in the ECRHS cohort.

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physical activity overtime) at *t* and lung function at *t* as the causal effect of physical activity on lung function, and the association between lung function at *t*-1 and physical activity at *t* as the causal effect of lung function on physical activity.

The following variables were selected as time-fixed confounders in the analyses: sex, education, age, age-squared, height, occupation, the AHEI-2010 score, childhood respiratory infection and centre. The following variables were selected as time-dependent confounders in the analyses: number of pack-years smoked, second-hand smoke exposure and weight at *t*-1. As the inclusion of BMI and menopausal status may compromise statistical power because of their correlation with weight and age/age-squared, respectively, and the inclusion of occupational exposures compromised statistical power because of high missingness, the three variables were only considered as covariates in a sensitivity analysis.

We used generalized SEM (exploratory approach based on logistic and linear regression models) to test the existence of the hypothesised relationships (i.e. "paths") depicted in Fig 1, and more particularly to investigate the bi-directionality of the association between physical activity and lung function, controlling for time-fixed and time-varying confounders [8]. The gsem command in STATA was used (more details are provided in the online S1 File). The association of physical activity with lung function was measured by the difference in expected lung function (β); the association of lung function with physical activity was measured by the odds ratio (OR).

We used MSM, an approach from the causal inference framework, to investigate whether the potential effect of physical activity on lung function remains after correcting for potential reverse causation (i.e. the potential effect of previous lung function on physical activity that may be suggested by the use of SEMs) and time-dependent confounding. MSMs were applied using inverse probability weighting, which inherently corrects for "cumulative confounding" throughout time, to allow the estimation of the *causal* effect of physical activity on lung function [9] by mimicking a hypothetical randomized experiment via the creation of a pseudo-population in which exposed and non-exposed subjects are exchangeable within levels of the available confounders [10] (more details, including STATA codes are provided in the online S1 File). The effect of physical activity on lung function was measured by the β coefficient.

As sensitivity analyses: (1) we used weight truncation (i.e. we reset the value of weights greater than the 95th percentile to the 95th percentile value and the value of weights lower than the 5th percentile to the 5th percentile value)., and (2) those who had avoided vigorous exercise because of wheezing or asthma at ECRHS II, as their inclusion may lead to an overestimation of the true protective effect of physical activity on lung function; (3) we repeated the MSMs analyses by restricting the study population to consistently active smokers throughout the follow-up (i.e. subjects who had reported current smoking at ECRHS I, II and III), and (4) by considering frequency of physical activity ($\leq$once a month, 1–3 times per week and $\geq$4 times per week) and duration per week ($\leq$30 minutes per week, 1–3 hours per week and $\geq$4 hours per week) as exposures of interest, in order to check the presence of a linear dose-response relationship between physical activity and lung function.

Analyses were conducted using STATA v14.0.

Ethics statement

Ethical approval from the appropriate ethics committees was obtained by all centres participating in the ECRHS: Regional Committees for Medical and Health Research Ethics (REK Vest, number 2010/759, date: 22nd march 2010) for Bergen (Norway); Ethik-Kommission der Bayerischen Landesärztekammer (number 10015, date: 8th June 2010) for Hamburg and Erfurt (Germany); Comité Ético de Investigación Clínica del Instituto Municipal de Asistencia

Sanitaria (number 2009/3500/l, date: 22nd June 2010) for Barcelona, Galdakao, Albacete, Oviedo and Huelva (Spain); The National Bioethics Committee (NBCI, ref: VSNb2011090016/03.11, date: 30th January 2015) for Reykjavik (Iceland); Regionala etikprövningsnämnden (number 2010/432, date: 12th January 2011 and number 2010/068, date: 24th March 2010) for Gothenburg, Umea and Uppsala (Sweden); Research Ethics Committee (REC, ref: 11/LO/0965, date: 7th July 2011) for Ipswich and Norwich (UK); Comité de protection des personnes Sud Est V (ref: 11-CHUG-03, date: 3rd March 2011) for Bordeaux, Grenoble, Montpellier and Paris (France). Written consent was obtained from all participants.

Results

Table 1 shows the main characteristics of the 753 participants included in the study (mean age at ECRHS II: 41 years; female: 46%). Between 31% and 38% of these individuals were considered physically active over the study period. Compared to the subjects included in the study population, those excluded were more likely to be women and to report an unknown/other occupation, otherwise they were similar in terms of age, lung function, physical activity, smoking and other characteristics (S1 Table in online S1 File).

Using generalized SEMs, positive associations of physical activity on lung function parameters were found at both ECRHS II and III (difference in expected FEV_1 (95%CI), active versus non-active: 53 mL (12, 94) and 43 mL (1, 85); difference in expected FVC (95%CI), active versus non-active: 49 mL (0, 98) and 50 mL (6, 106); see Fig 2). We only identified positive associations of lung function at ECRHS I on physical activity at ECRHS II (OR (95% CI), 500 mL increase in FEV_1 1.34 (1.09, 1.66); OR (95% CI), 500 mL increase in FVC: 1.23 (1.04, 1.46); see Fig 2).

The inclusion of BMI, menopausal status and occupational exposures as additional covariates did not substantially alter our results.

Using MSMs, strong positive effects were found between being physically active and having higher lung function levels (difference in expected FEV_1 (95% CI), active versus non-active: 58 mL (21–95); difference in expected FVC (95% CI), active versus non-active: 83 mL (36–130); see Fig 3). Similar effects were found when the MSM analyses were repeated using truncated weights, suggesting that the magnitude of time-dependent confounding is relatively low (Fig 3). When we repeated the MSM analysis including only the 336 subjects who had consistently reported being current smokers at ECRHS I, II and III, the estimated effects remained stable although results lost statistical significance (Fig 3). When the MSM analyses were conducted to investigate the effects of frequency and duration of physical activity on lung function, strong linear positive relationships were found (Fig 4).

Discussion

This is the first longitudinal study among adult current smokers to investigate and report a positive bi-directional association between physical activity and lung function, although this finding was exploratory and not consistent throughout the study's follow-up. The notion that lung function impacts physical activity likely comes from the fact that exercise limitation is a well-known consequence of respiratory conditions [11]. However, people with normal lung function (as is the case for most of our sample) have a wide range of 'potential' physical activity levels, and as physical activity is a behaviour, it is affected by many more factors other than lung function alone [12]. Thus, it is possible that the bi-directionality between physical activity and lung function can only be properly studied in other samples covering wider (i.e. including the lowest) ranges of both parameters, such as in clinical studies.

Table 1. Description of the study population (n = 753).

Time of assessment*	ECRHS I	ECRHS II (baseline)	ECRHS III
Outcomes of interest (also considered as time-varying confounders)			
FEV_1 (mL), m (SD)	3.7 (0.8)	3.5 (0.7)	2.9 (0.7)
FVC (mL), m (SD)	4.5 (0.9)	4.3 (0.9)	4.0 (0.9)
Exposure of interest			
Physical Activity			
Active (%)	-	30.7	38.0
Frequency (%)			
≤1 a month		49.7	44.1
1–3 times a week	-	40.1	41.0
≥4 times a week		10.2	14.9
Duration (%)			
≤30 min		48.1	46.2
1–3 hours	-	39.0	34.1
≥4 hours		12.9	19.7
Time-varying confounders			
Number of pack-years smoked, m (SD)	13.1 (11.4)	21.5 (17.1)	
Passive smoking (%)	78.8	65.2	
Weight (kg), m (SD)	70.5 (13.3)	74.1 (14.7)	
Menopausal status in women (%)			
Pre-menopausal	96.1	84.2	
Post-menopausal	3.9	15.8	
Time-fixed confounders			
Sex (%)			
Female	45.5		
Male	54.5		
Education (%)			
<17 years	22.1		
17–20 years	34.6		
>20 years	43.3		
Age (years), m (SD)		41.4 (7.0)	
Height (cm), m (SD)	170.2 (8.9)	-	
Occupation (%)			
Management/professional/non-manual		26.6	
Technical/professional/non-manual		18.9	
Other non-manual		23.9	
Skilled manual		13.6	
Semiskilled/unskilled manual		13.0	
Other/unknown		4.1	
Alternative healthy eating index-2010[±], m (SD)		50.4 (8.1)	50.4 (12.4)
Respiratory infection during childhood (%)	10.4		
Occupational exposure to dust, gas/fumes or pesticides during follow-up (%)		53.4	

m: mean; SD: standard deviation

*As shown in Fig 1, outcome data were considered at ECRHS I, II and III, exposure data were considered at ECRHS II and III, time-varying confounder data were considered at ECRHS I and II, and time-fixed confounder data were considered only once (i.e. when available).

[±] The AHEI-2010 score was derived at ECRHS III for sixteen centres; two additional centres had dietary data at ECRHS II only.

https://doi.org/10.1371/journal.pone.0237769.t001

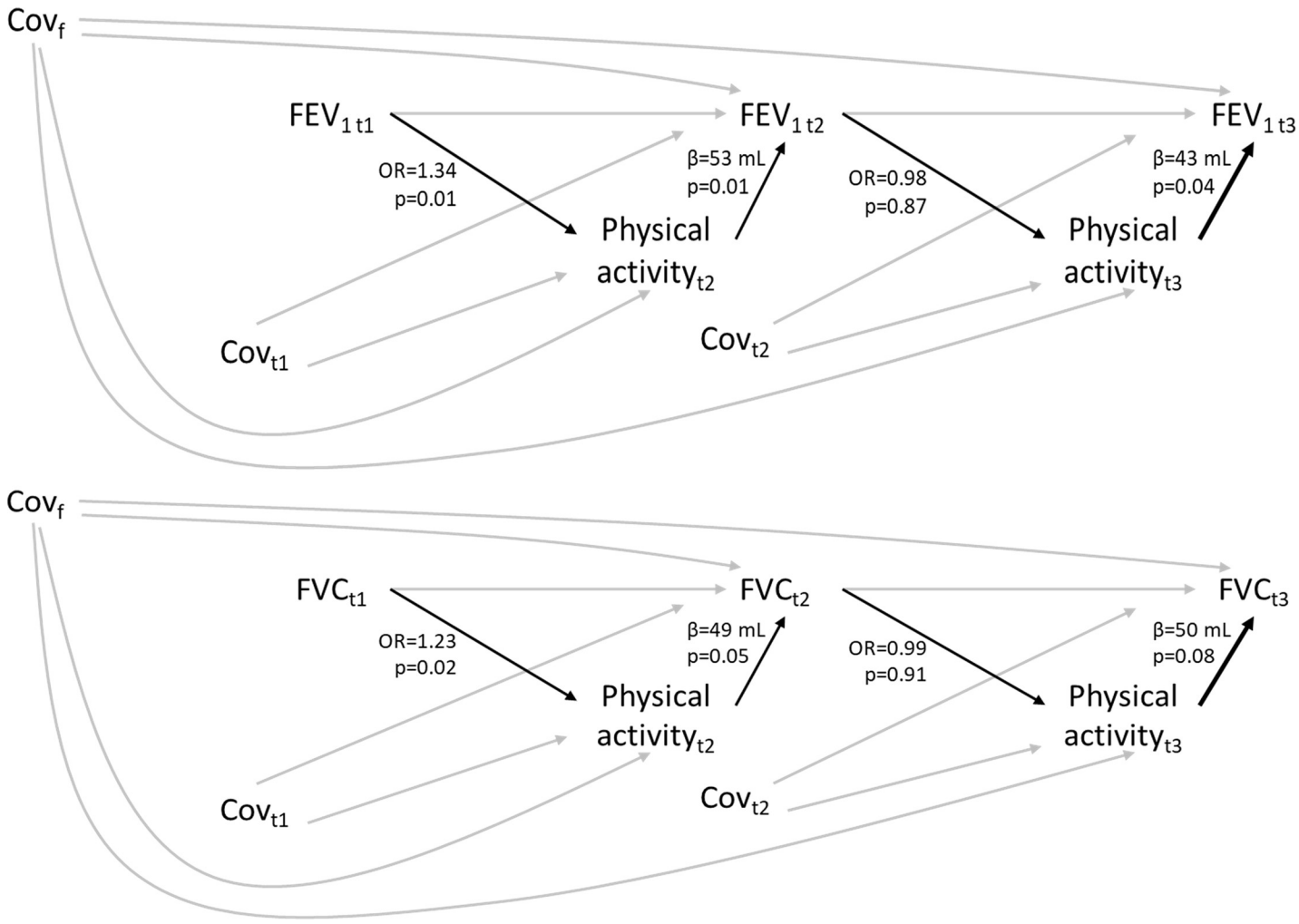

Fig 2. Associations of physical activity with lung function over time estimated using SEMs in the ECRHS cohort. Cov $_t$ (time-varying confounders): number of pack-years smoked, passive smoking exposure, weight. Cov $_f$ (time-fixed confounders): sex, education, age, age-squared, height, occupation, AHEI-2010 score, respiratory infection in childhood, centre. NB: The inclusion of BMI (instead of weight), menopausal status (in addition to age and age-squared), and occupational exposures compromised statistical power without substantially altering the results, thus they were not considered as covariates in the final models. β: difference in the expected lung function measure comparing active versus non-active individuals. OR: odds ratio comparing the risk of being active versus non-active for each 500 mL increase in lung function measures.

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This study also found a positive effect between physical activity and lung function after removing potential time-dependent confounding and taking into account the association between previous lung function and physical activity.

Several studies have found positive associations between physical activity [1–3,13–21] and lung function levels in adults but most of them were cross sectional [15–17] or conducted in specific populations such as COPD patients [18] or adults with asthma [19]. A few prospective studies suggested a beneficial effect of physical activity on lung function in healthy adults [2,13,20,21] or in the general population [1,3,14], although results are inconsistent in terms of assessment of physical activity, length of follow-up or adjustment for potential confounders. The evidence linking regular physical activity and improved lung function is growing and appears to suggest stronger associations among current smokers [1,2]. Our results are consistent with the results from a previous MSM analysis conducted in the Copenhagen City Heart Study [3] and overcome some limitations by including dietary data. Our study also goes

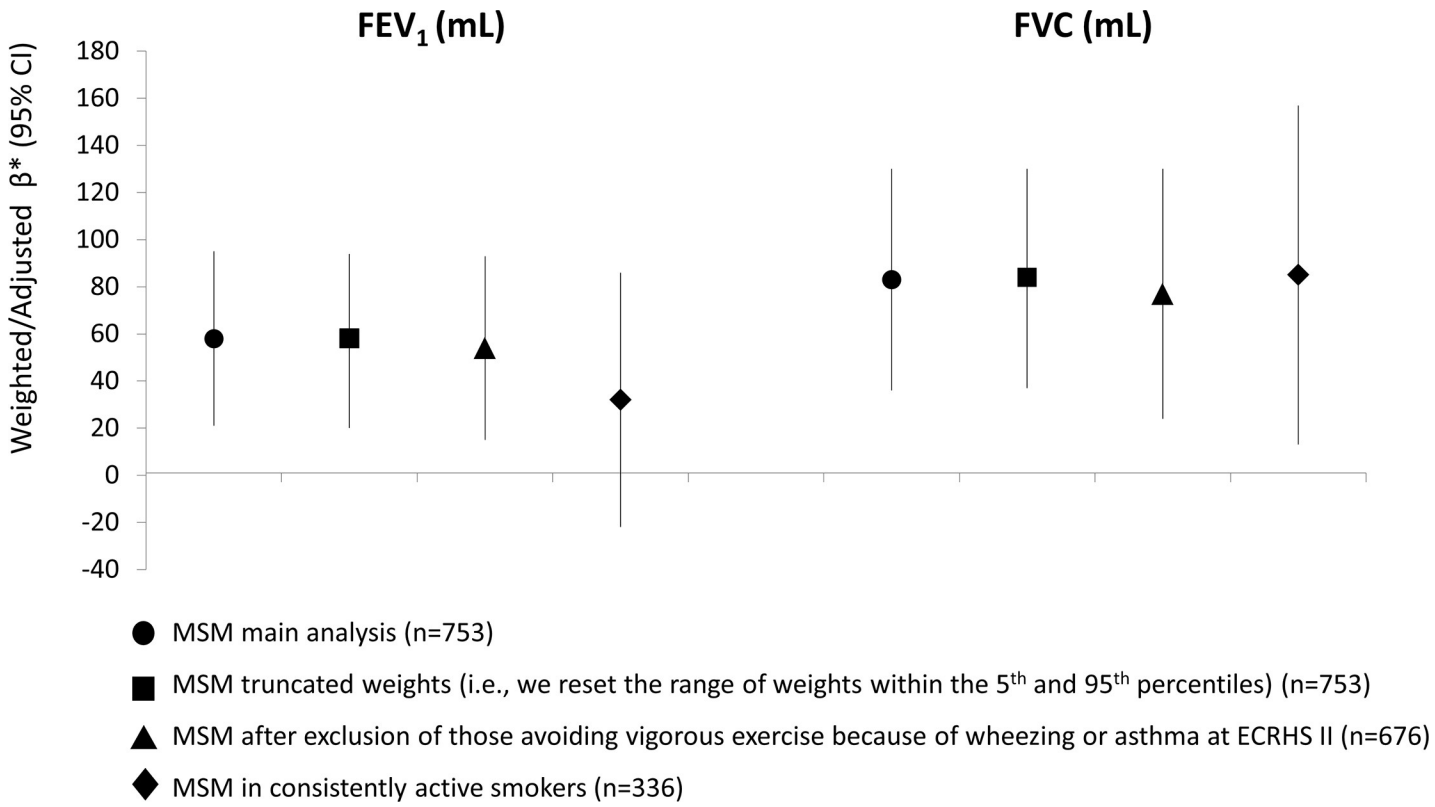

Fig 3. Effects of physical activity on lung function estimated using MSMs (main and sensitivity analysis) in the ECRHS cohort. β: difference in the expected lung function measure comparing active versus non-active individuals. *Models included. number of pack-years smoked, passive smoking exposure, weight, sex, education, age, age-squared, height, occupation, AHEI-2010 score, respiratory infection in childhood), and centre.

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beyond that previous study by investigating more thoroughly reverse causation (i.e. studying also the potential effect of lung function on physical activity) and including a more geographically diverse population with a wider range of exposures, outcomes and covariates.

The use of MSMs and the fact that the results were robust to sensitivity analyses and are consistent with the literature [1,2] supports causal interpretation of the protective effect of physical activity on lung function.

A major strength of this study is the use of two complementary approaches to address a methodologically challenging research question. Other strengths include its longitudinal design, population-based nature, broad geographical representation of participants, and the availability of repeated measurements for outcome, exposure, and relevant confounders—some of which (e.g. diet) were not considered before.

This study's main limitation is that the design of the ECRHS, with questionnaires administered ten-years apart, allowed only two cross-sectional estimations between physical activity and lung function, which may not allow time-dependent confounding to be fully addressed. However, it is worth mentioning that at the time of their lung function measurement, ECRHS subjects were asked about their *usual* physical activity. Hence assuming that physical activity at time t precedes lung function at time t seems reasonable. Moreover, as similar results were found after excluding those who had reported that they 'avoided vigorous exercise because of wheezing/asthma', suggesting that the positive effects found between physical activity and lung function are not driven by these subjects, residual time-dependent confounding is less likely to be an explanation. Another potential limitation is the information bias due to the

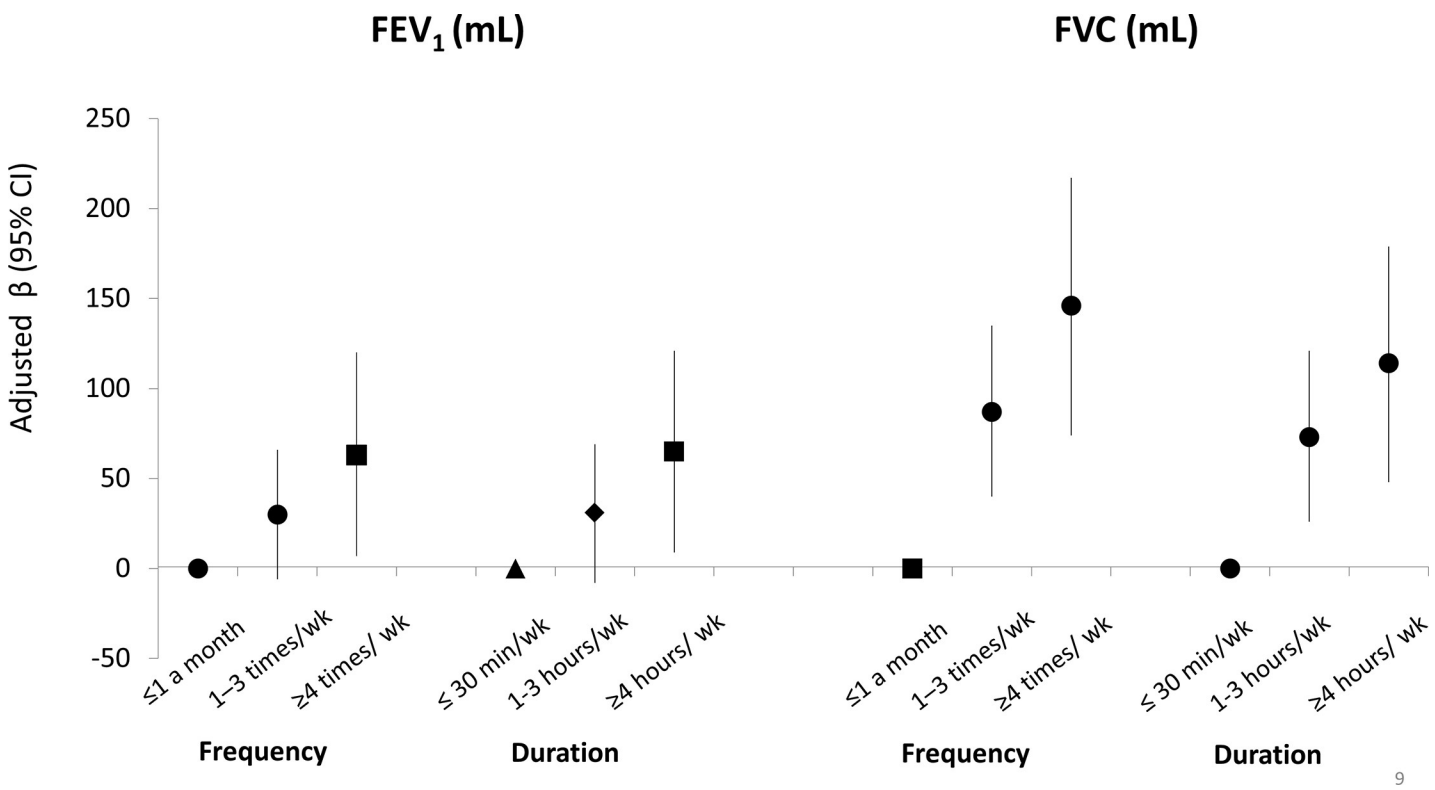

Fig 4. Effects of frequency and duration of physical activity on lung function, estimated using MSMs in the ECRHS cohort. β: difference in the expected lung function measure. *Models included number of pack-years smoked, passive smoking exposure, weight, sex, education, age, age-squared, height, occupation, AHEI-2010 score, respiratory infection in childhood), and centre.

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misclassification of physical activity, although this potential error is likely to have been non-differential with respect to lung function, and thus would be expected to bias effect estimates towards the null. No information was available for *moderate* physical activity, which may be more beneficial for lung function [2], and no repeated information was available on body composition (only body weight). We cannot rule out the possibility that the exclusion of ECRHS participants without complete information for this specific analysis might have biased our findings. However, our analyses showed no relevant differences between the included and excluded subjects. Finally, although many known confounders were accounted for, we cannot rule out residual/unmeasured confounding, e.g. from socioeconomic status (adjusted for using years of education) or dietary habits (only assessed once).

In conclusion, our results suggest bi-directional causation and support a true protective effect of physical activity on lung function in smokers, after accounting for reverse causation and time-dependent confounding.

Supporting information

S1 File.
(DOCX)

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PLOS ONE

RESEARCH ARTICLE

Chronic bronchitis without airflow obstruction, asthma and rhinitis are differently associated with cardiovascular risk factors and diseases

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Abstract

Background and objectives

Cardiovascular and respiratory diseases can frequently coexist. Understanding their link may improve disease management. We aimed at assessing the associations of chronic bronchitis (CB), asthma and rhinitis with cardiovascular diseases and risk factors in the general population.

Methods

We used data collected in the Gene Environment Interactions in Respiratory Diseases study, an Italian multicentre, multicase-control study. Among 2463 participants (age 21–86, female 50%) who underwent standardized interviews, skin prick and lung function tests, we identified 254 cases of CB without airflow obstruction, 418 cases of asthma without CB, 959 cases of rhinitis alone, and 832 controls. The associations of respiratory diseases with reported cardiovascular risk factors (lifestyles, hypertension, dyslipidaemia), heart disorders (myocardial infarction, coronary thrombosis, angina, aorta or heart surgery) and intermittent claudication were estimated through relative risk ratios (RRR) by multinomial logistic regression models.

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Competing interests: The authors have declared that no competing interests exist.

Abbreviations: BMI, body mass index; CB, chronic bronchitis; COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; GEIRD, Gene Environment Interactions in Respiratory Diseases; LLN, lower limit of normal; PD_{20}, provocative dose causing a 20% decrease in FEV_1; RRR, relative risk ratios.

Results

Compared to controls, CB cases were more likely to be heavy smokers, alcohol consumers, physically inactive, and to suffer from hypertension or dyslipidaemia; rhinitis cases were less obese but more likely to have hypertension. Asthma was significantly associated with current smoking. After adjusting for cardiovascular risk factors, heart disorders were associated with CB (RRR[95%CI]: 1.58[1.12–2.22]) and rhinitis (1.35[0.98–1.85]) and intermittent claudication was associated with CB (3.43[2.52–4.67]), asthma (1.51[1.04–2.21]) and rhinitis (2.03[1.34–3.07]).

Conclusions

CB, asthma and rhinitis were associated with cardiovascular risk factors and diseases. In particular, CB shared with cardiovascular diseases almost all risk factors and was strongly associated with a higher risk of heart disorders and intermittent claudication.

Introduction

Several previous studies have shown a significantly increased risk of cardiovascular diseases in COPD and clinicians have long recognized that cardiovascular diseases are the major contributor to morbidity and mortality in patients with COPD [1,2].

Other respiratory diseases have been associated with cardiovascular diseases. Large population studies showed that patients with chronic bronchitis (CB), a respiratory condition associated with a decline in lung function [3], have increased respiratory, cardiovascular and all-cause mortality [4–7].

Data on the association between asthma and cardiovascular diseases are conflicting [8–14], and few studies have addressed the relationship between rhinitis and cardiovascular diseases [15–17].

Previous research on the intriguing coexistence of respiratory and cardiovascular diseases generally focused on a single respiratory disorder, and few studies investigated the cardiovascular risk factors associated with airway illnesses. With this in mind, we aimed to investigate the association of cardiovascular diseases and cardiovascular risk factors with CB, asthma and rhinitis, by analysing data collected in the population-based Gene Environment Interactions in Respiratory Diseases (GEIRD) study.

Methods

Study design and selection of the cohort

GEIRD is a two-stage multicentre, multicase–control study carried out in Italy (www.geird.org) [18]. In the first stage, new random samples of adults (GEIRD 20–64 years) and elderly subjects (GEIRD 65–85 years), male/female = 1/1, or pre-existing randomly sampled cohorts from the general population (ISAYA, ECRHS Italy and ECRHS III) were surveyed for respiratory symptoms between 2006 and 2010 using a postal questionnaire, as reported in Table 1 and described elsewhere [19]. Overall, 14,513 subjects from the centres of Verona, Pavia, Torino, Ancona, Sassari answered the questionnaire (response rate: 59%). All the subjects who reported symptoms suggestive of asthma or chronic bronchitis, a random sample (30%) of the subjects who reported rhinitis or hay fever and a random sample (40%) of the subjects who did not report respiratory symptoms, diagnoses or hospitalizations were invited to clinics. Additionally, a sample of 439 subjects from Palermo was invited. Overall, 7,025 subjects were

Table 1. Study population by centre and cohort.

Center	Cohort	Invited to stage 2	Participating in stage 2	Included in the analysis	Females (%)	Age, years (mean ± SD)
Verona	ECRHS III	185	98	95	47.4	54.2 ± 7.6
	GEIRD 20–64	2,961	1,329	1,165	52.2	44.5 ± 10.2
	ISAYA					
	ECRHS Italy					
	GEIRD 65–84	591	132	97	34.0	71.8 ± 2.8
Turin	ECRHS III	178	76	69	55.1	53.9 ± 6.3
	GEIRD 20–64	589	359	282	52.1	46.8 ± 10.3
Pavia	ECRHS III	186	95	86	53.5	57.2 ± 6.7
	GEIRD 20–64	489	241	204	62.3	50.9 ± 11.0
Ancona	GEIRD 20–44	575	99	91	53.8	42.3 ± 5.3
Sassari	GEIRD 65–84	439	189	122	32.0	74.2 ± 4.3
	ISAYA	393	230	207	49.3	45.0 ± 6.9
Palermo	GEIRD 65–84	439	63	45	33.3	75.1 ± 4.7
Overall		**7,025**	**2,911**	**2,463**	**50.7**	**49.5 ± 12.8**

ECRHS, European Community Respiratory Health Survey; GEIRD, Gene Environment Interactions in Respiratory Diseases; ISAYA, Italian Study on Asthma in Young Adults.

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invited to GEIRD stage-2 [20]. Between 2007 and 2015, the participants in GEIRD stage-2 were 2,911 subjects (participation rate: 41%). In all centres, clinical examinations were carried out following standardised protocols. Ethical approval was obtained from the following ethics committees: Verona, Comitato Etico per la Sperimentazione dell'Azienda Ospedaliera Istituti Ospitalieri di Verona; Turin, Comitato Etico dell'Azienda Sanitaria Locale TO/2 di Torino; Pavia, Comitato di Bioetica della Fondazione IRCCS Policlinico San Matteo di Pavia; Ancona, Comitato Etico dell'Azienda Ospedaliero-Universitaria Ospedali Riuniti di Ancona; Sassari, Comitato di Bioetica dell'Azienda Sanitaria Locale di Sassari; Palermo, Comitato Etico dell'Azienda Ospedaliera Ospedali Riuniti Villa Sofia Cervello. Written consent was obtained from each participant.

Clinical measurements

Forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) were measured according to the American Thoracic Society reproducibility criteria [21]. Lung function values were expressed as a percentage of predicted values, and the lower limit of normal LLN for the FEV_1/FVC was calculated according to Quanjer [22]. Spirometry was performed again 10 min after the administration of 400 μg salbutamol in subjects with FEV_1/FVC <70% or <LLN. The subjects with FEV_1/FVC ≥70% and ≥LLN underwent the methacholine challenge, according to a standardized protocol [23]. A subject's test was positive if FEV_1 decreased by 20% at a maximum cumulative dose ≤1 mg methacholine (PD_{20} ≤1).

The subjects were skin tested for a panel of 14 aeroallergens [20]. A subject was considered to be atopic if positive to one or more of the tested allergens.

Identification of cases and controls in clinics

Based on the symptoms reported and the results of the clinical tests, 2,463 subjects were hierarchical classified into four groups: CB (n = 254), asthma (n = 418), rhinitis (n = 959), and controls (n = 832). The cases and control groups were defined as follows:

- **cases of chronic bronchitis** (CB): subjects with self-reported cough and phlegm for the most of days in 3 consecutive months, during 2 years, with post-bronchodilator $FEV_1/FVC \geq 70\%$ and $\geq$LLN.
- **cases of asthma**: subjects without CB who had 1) self-reported asthma, plus one among 1.1) having had an asthma attack in the last 12 months, 1.2) current use of medications for asthma; or 2) asthma-like symptoms or use of medicines for breathing problems in the last 12 months, plus one among 2.1) $PD_{20} \leq 1$ mg, 2.2) pre-bronchodilator $FEV_1/FVC < 70\%$ or $<$LLN with a positive reversibility test (i.e. FEV_1 improvement $\geq$ 12% and $\geq$ 200mL after 400μg of salbutamol);
- **cases of rhinitis**: subjects without CB and asthma who had one among 1) lifetime nasal allergies, including 'hay fever'; 2) lifetime problem with sneezing, or a runny or a blocked nose (without cold/flu); 3) recurrent nasal/eye symptoms in the presence of dust, pollens or animals.
- **controls**: subjects who were not cases and had both (i) pre-bronchodilator $FEV_1/FVC \geq 70\%$ and $\geq$LLN; and (ii) $FEV_1 >$ 80% predicted.

Sixty-eight subjects with COPD, defined as having both persistent respiratory symptoms (dyspnoea, cough, and/or sputum production) and airflow limitation (post-bronchodilator $FEV_1/FVC < 70\%$ or $<$LLN), and 380 subjects who did not correspond to any of the definitions above or with missing values on key information were excluded from the analyses.

Cardiovascular diseases

Two different self-reported doctor-diagnosed cardiovascular conditions were considered [24]:

1. **heart disorders**, defined as having any among coronary heart disease ('Did a physician tell you that you suffer from: myocardial infarction, coronary thrombosis, or angina?'), heart/aortic surgery ('Have you ever undergone heart or aortic surgery?').
2. **intermittent claudication**. A subject was considered to have intermittent claudication if he/she answered yes to the question: 'Do you get a pain or discomfort in your legs when you walk?', plus he/she reported that it usually disappeared in 10 min or less when standing still [25]. Intermittent claudication was adopted as proxy of peripheral arterial disease [26].

Covariates

Information on the following variables was collected during the clinical interview and was taken into account for the analyses: age, gender, school education as a proxy of the socio-economic status (low if had completed full-time education before the age of 16), smoking habits (lifetime non-smoker, ex-smoker, current smoker), daily alcohol intake (lifetime non-consumer, moderate ($\leq$15 g/day), high (>15 g/day)), sedentary life (usually doing physical exercise less than once per month), and self-reported diabetes, hypertension or dyslipidaemia (high levels of cholesterol or triglycerides).

Statistical analysis

The subjects' characteristics were summarized as percentages or means (SD). The Pearson's chi-squared test, Fisher's exact test and ANOVA were used to test differences across cases and controls ($\alpha = 0.05$). The associations of cardiovascular diseases/risk factors with respiratory diseases were estimated through relative risk ratios (RRR) obtained by multinomial logistic

regression models using the case/control indicator (0 = control, 1 = chronic bronchitis, 2 = asthma, 3 = rhinitis) as the dependent variable and cardiovascular diseases/risk factors as the main independent variable. Three models were fitted to the data: (i) adjusted for age and sex; (ii) further adjusted for smoking habits, alcohol consumption, body mass index (BMI, in categories <25, 25–30 and >30 kg/m^2), school education and physical activity; (iii) further adjusted for the comorbidity indicators (diabetes, hypertension, dyslipidaemia). Centre was considered a clustering factor and cluster-robust standard errors were used.

Statistical analyses were performed with STATA 13.1 (Stata Corp. College Station, TX, USA).

Results

Overall, 2,463 subjects were classified as cases or controls and included in the study. The age ranged from 21 to 86 years, and 50.7% (n = 1,249) were females (Table 1).

As a result of our hierarchical definitions, cases of CB could also be affected by asthma (n = 115, 45.3%) as well as rhinitis (n = 205, 80.7%), and cases of asthma could also be affected by rhinitis (n = 354, 84.7%). Cases of asthma and CB had a significantly lower FEV_1 and FEV_1/FVC ratio than controls (Table 2). The proportion of subjects with atopy was significantly higher among cases of CB, asthma and rhinitis (57.8, 79.0 and 62.5%, respectively) compared to the control group (26.0%).

Table 2. Distribution of risk factors, clinical characteristics and lung function measurements by cases-control status.

	Chronic bronchitis	Asthma	Rhinitis	Controls	p-value
	n = 254	n = 418	n = 959	n = 832	
Sex (females)	145 (57.1)	194 (46.4)	496 (51.7)	414 (49.8)	0.048
Age (year)	48.4 ± 12.8	46.4 ± 12.2	49.7 ± 13.0	51.1 ± 12.6	<0.001
Low education	65 (25.7)	64 (15.6)	152 (16.0)	161 (19.5)	0.001
Smoking habits					<0.001
never smokers	112 (44.1)	196 (46.9)	490 (51.3)	430 (54.7)	
ex-smokers	58 (22.8)	125 (29.9)	295 (30.9)	268 (32.3)	
current-smokers	84 (33.1)	97 (23.2)	171 (17.9)	133 (16.0)	
Alcohol consumption					0.005
no	133 (53.4)	231 (56.6)	594 (63.0)	543 (62.9)	
moderate (≤15 g/day)	66 (26.5)	115 (28.2)	222 (23.5)	210 (25.7)	
high (>15 g/day)	50 (20.1)	62 (15.2)	127 (13.5)	93 (11.4)	
Sedentary life	163 (64.2)	212 (50.8)	488 (51.2)	437 (52.5)	0.002
BMI					0.388
<25 kg/m^2	123 (50.6)	224 (54.9)	511 (55.8)	317 (50.2)	
25–30 kg/m^2	82 (33.7)	127 (31.1)	295 (32.2)	292 (35.1)	
>30 kg/m^2	38 (14.7)	57 (14.0)	110 (12.0)	122 (14.7)	
Diabetes	9 (3.5)	11 (2.6)	35 (3.7)	31 (3.7)	0.766
Hypertension	80 (31.6)	91 (21.8)	243 (25.4)	199 (24.0)	0.032
Dyslipidaemia	97 (38.2)	115 (27.7)	277 (29.0)	254 (30.5)	0.022
FEV_1 (L)	3.15 ± 0.87	3.24 ± 0.89	3.29 ± 0.83	3.32 ± 0.78	<0.001
FVC (L)	4.02 ± 1.09	4.20 ± 1.15	4.05 ± 1.03	4.04 ± 0.96	<0.001
FEV_1/FVC ratio (%)	78.6 ± 8.6	77.4 ± 6.8	81.3 ± 6.3	82.4 ± 6.0	<0.001
FEV_1 (% of predicted)	96.2 ± 14.7	94.4 ± 14.0	100.9 ± 12.9	103.2 ± 12.3	<0.001
Atopy	122 (57.8)	278 (79.0)	498 (62.5)	177 (26.0)	<0.001

Data are presented as n (%) or mean ± standard deviation.

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Table 3. Relative risk ratios, with 95% confidence intervals, for the associations of demographics, cardiovascular risk factors and comorbidities with CB, asthma, and rhinitis.

	Chronic bronchitis vs. Controls RRR (95% CI)	Asthma vs. Controls RRR (95% CI)	Rhinitis vs. Controls RRR (95% CI)
Sex (female vs. male)	**1.63 (1.31–2.04)**	0.91 (0.76–1.10)	1.09 (0.96–1.24)
Age (per year increase)	**0.96 (0.94–0.98)**	**0.96 (0.95–0.98)**	0.99 (0.98–1.00)
Education (low vs. high)	**1.78 (1.24–2.54)**	1.00 (0.66–1.50)	0.82 (0.66–1.02)
Smoking habits			
ex vs. never smokers	0.78 (0.59–1.03)	1.15 (0.91–1.46)	1.01 (0.84–1.22)
current vs. never smokers	**2.00 (1.41–2.84)**	**1.52 (1.20–1.94)**	1.16 (0.98–1.37)
Alcohol consumption			
moderate vs. no	1.27 (0.87–1.86)	0.98 (0.74–1.28)	0.90 (0.70–1.16)
high vs. no	**2.55 (1.61–4.03)**	1.44 (0.99–2.11)	1.22 (0.92–1.61)
Sedentary vs. active life	**1.59 (1.05–2.42)**	1.02 (0.83–1.26)	0.96 (0.73–1.26)
BMI			
25–30 vs. <25 kg/m^2	1.00 (0.78–1.30)	0.91 (0.66–1.27)	**0.86 (0.79–0.94)**
>30 vs <25 kg/m^2	0.88 (0.64–1.21)	0.99 (0.75–1.30)	0.73 (0.52–1.03)
Diabetes (yes vs.no)	0.91 (0.50–1.67)	0.82 (0.38–1.79)	1.12 (0.78–1.61)
Hypertension (yes vs.no)	**1.90 (1.14–3.15)**	**1.41 (1.06–1.89)**	**1.42 (1.13–1.77)**
Dyslipidaemia (yes vs.no)	**1.66 (1.19–2.31)**	1.02 (0.84–1.24)	0.95 (0.83–1.09)

Adjusted for all the variables included in the table. RRR, relative risk ratio

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The groups differed in the distribution of sex and age, as well as of most of lifestyle variables and cardiovascular risk factors: education, smoking habits, alcohol consumption, physical activity, hypertension, dyslipidaemia (Table 2). In particular, CB was significantly associated with female sex (RRR 1.63, 95%CI: 1.31–2.04), younger age (0.96, 0.94–0.98), and low education (1.78; 1.24–2.54) (Table 3).

Current smoking (2.00, 1.41–2.84), high alcohol consumption (2.55, 1.61–4.03) and sedentary life (1.59, 1.05–2.42) were also associated with an increased risk of CB. The risk of having asthma was higher in younger subjects (0.96, 0.95–0.98) and in current smokers (1.52, 1.20–1.94). The risk of having rhinitis was lower in overweight subjects (0.86, 0.79–0.94). Hypertension was significantly associated with CB (1.90, 1.14–3.15), asthma (1.41, 1.06–1.89) and rhinitis (1.42, 1.13–1.77). Dyslipidaemia was associated with CB (1.66, 1.19–2.31).

The crude prevalence of heart disorders was higher among cases of CB and rhinitis (14.2% and 12.1%) compared to controls and asthma cases (10.1% and 9.3%) (Fig 1).

After adjustment for all cardiovascular risk factors and comorbidities (Fig 2, model 3), heart disorders were significantly associated with CB (RRR, 95%CI: 1.58, 1.12–2.22; p = 0.009). A borderline association was also detected between heart disorders and rhinitis (RRR, 95%CI: 1.35, 0.98–1.85; p = 0.066).

The prevalence of intermittent claudication was higher in all the cases groups (10.6%, 4.8%, 6.0% in CB, asthma, and rhinitis groups, respectively) than in controls (3.3%) (Fig 1). In the fully adjusted model (Fig 2, model 3), intermittent claudication was significantly associated with a 3.5-fold higher risk of CB (RRR 3.43, 95%CI: 2.52–4.67; p<0.001), a 2-fold higher risk of rhinitis (2.03, 1.34–3.07; p<0.001), and a 1.5-fold higher risk of asthma (1.51, 1.04-2-21; p = 0.032).The associations were confirmed when using different sets of adjustment variables (Fig 2, models 1 and 2).

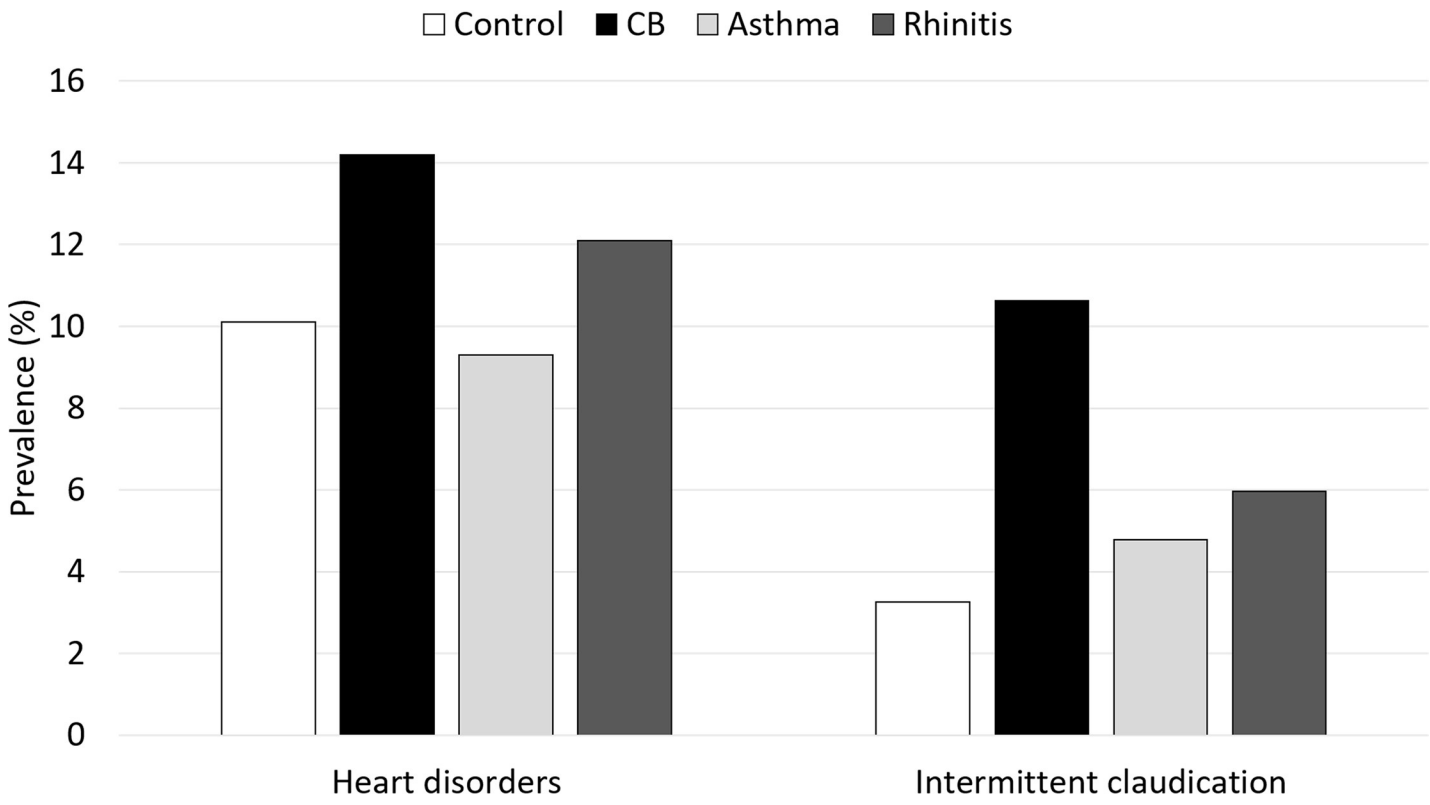

Fig 1. Prevalence of heart disorders and intermittent claudication in subjects with CB, asthma, rhinitis, and controls.

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Discussion

In the present analysis of data from the general population, we found that heart disorders and intermittent claudication, an indicator of symptomatic peripheral artery disease, were both strongly associated with CB and, to a minor extent, with rhinitis; intermittent claudication was also associated with asthma. Our data also indicate that cardiovascular risk factors are frequently associated with respiratory illnesses. Particularly, our findings support that unhealthy lifestyles (smoking, high alcohol consumption and sedentariness), hypertension and dyslipidaemia may predict a greater risk of CB.

Our findings should be interpreted keeping in mind that, in this multicase-control study, we used a hierarchical classification of diseases, so that cases of chronic bronchitis may also suffer from asthma and rhinitis, and cases of asthma could also be affected by rhinitis. Furthermore we underline that control subjects were accurately selected on the basis of the absence of CB, asthma and rhinitis.

CB was strongly and independently associated with heart disorders. Of note, as cases of COPD were excluded from the analysis, all subjects reporting CB presented a preserved lung function. Our results are in agreement with previous studies, demonstrating an increased risk of coronary disease and mortality among subjects with symptoms of CB [4,27,28]. In the above mentioned studies, lung function test was not performed, so that CB population could include subjects with COPD, which is known to be associated with cardiovascular diseases [2]. Lange et al. [5] and Guerra et al.[3] demonstrated an association between CB without bronchial obstruction, and all-cause death, indirectly supporting the results from the present analysis.

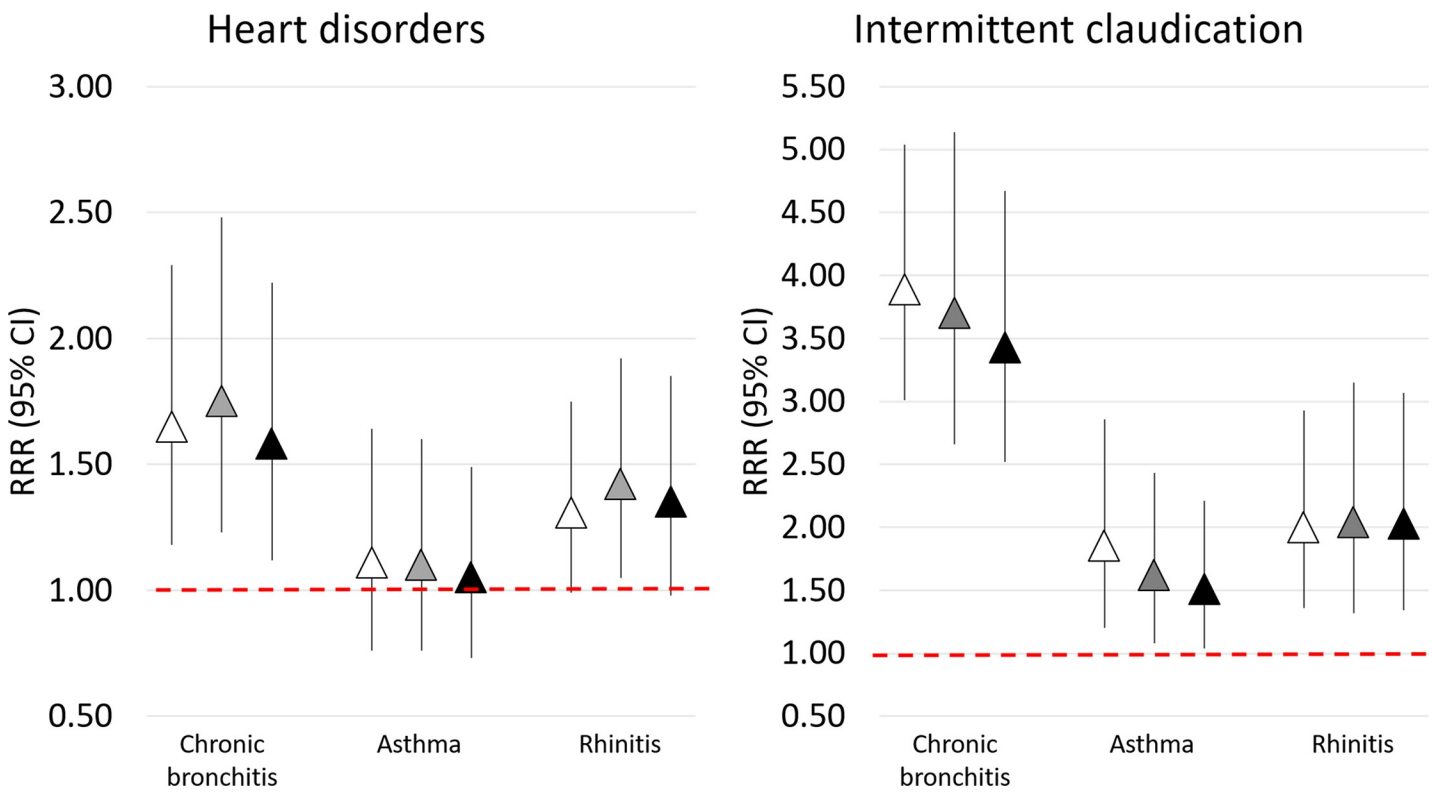

Fig 2. Relative risk ratios (RRR) with 95% CIs for the associations of CB, asthma, and rhinitis with heart disorders and intermittent claudication. Model 1 (white triangles): adjusted for age and gender; model 2 (grey triangles): adjusted for age, gender, school education, smoking status, alcohol consumption, BMI, physical activity; model 3 (black triangles): adjusted for variables in model 2 plus diabetes, hypertension, dyslipidaemia.

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This study is the first to report an association between CB and intermittent claudication, which remained significant after controlling for all the most important cardiovascular risk factors. These results suggest that CB may be an independent risk factor for atherosclerosis.

The lack of a significant association between asthma and heart disorders is in agreement with Schanen and colleagues, who reported that asthma is not a risk factor for coronary heart disease [10]. Nonetheless, some reports suggested an association of asthma with carotid atherosclerosis, coronary heart disease and stroke [9–14]. One possible explanation for the contrasting results is the fact that, differently from previous studies [9–12], we analysed asthma separately from CB, a disorder we found strongly associated with cardiovascular risk factors and diseases. Furthermore, in our study asthma was precisely characterized, whereas it was self-reported in some previous investigations [13], a fact that could introduce a bias of misclassification with COPD [29].

Extending the finding of our previous analysis that was carried out using data from the centre of Verona alone [16], subjects with rhinitis and/or asthma had an increased risk of suffering from intermittent claudication.

Few studies investigated the relationship between cardiovascular diseases and rhinitis [17,30]. In disagreement with our results, Hirsch et al. did not find a significant association between chronic rhino-sinusitis and post-morbid cardiovascular conditions [31].

The strong association of smoking with CB is not surprising [32], but we also found an increased risk of asthma among current smokers, in line with findings from others [33,34].

Our results do not support previous findings suggesting a relationship between smoking and prevalence of chronic rhinitis [35].

We found an association between dyslipidaemia and CB. Subjects with CB are more likely to be heavy smokers and alcohol consumers, which may be responsible of an altered lipid metabolism [36,37]. However, the association between dyslipidaemia and CB persisted after controlling for these confounders.

Another interesting result is that subjects with rhinitis were less likely to be overweight or obese, which was also observed in other two recent surveys [38,39].

Differently from previous studies showing an association between obesity and the risk of asthma [40], we found no relationship between asthma and BMI.

In agreement with previous studies [41], elevate alcohol consumption was independently correlated with CB. Alcohol acts systemically with various mechanisms (alteration of immunity and promotion of systemic inflammation) [42], that may be involved in both the respiratory and cardiovascular damage.

Our finding of a relationship between rhinitis and hypertension is consistent with a previous study by Kony et al. [43]. Our data also indirectly support another case-control study that suggested an increased incidence of hypertension among subjects with rhino-sinusitis [31]. While the association between COPD and hypertension has widely been described [2], our study is the first to report a strong positive association between CB and this risk factor. Our data also show the increased risk of arterial hypertension among asthmatic subjects, in agreement with previous findings from large population based studies [9,44].

The nature of the association of cardiovascular disorders with rhinitis and CB remains speculative and several mechanisms, such as infection [45–47] and inflammation, may play a role. The association of atherosclerosis with respiratory diseases could also be caused by the inherent susceptibility of some subjects to specific inflammatory pathways. There is evidence that patients with rhino-sinusitis [48] and CB [3] have higher levels of C-reactive protein, a predictive marker of coronary heart disease [49].

Asthma and CB are both characterized by chronic inflammation in the lung, even though the nature of the inflammation differs between the two disorders [8,50]. The different types of inflammation probably result in distinct pathology, clinical manifestation [8], and could differently influence the development of cardiovascular comorbidities.

Finally, reversal causation could not be excluded, since our study design does not consent to assess the temporal relationship between respiratory disorders, cardiovascular diseases and risk factors. Subjects affected by cardiovascular disease and hypertension often use medication that might induce respiratory symptoms (such as cough) or disorders (such as rhinitis) [51]. However the association between rhinitis, CB, asthma and hypertension was not modified after adjusting for antihypertensive treatment (data not shown).

The strength of our analysis is based on the standardized protocol which allowed a precise definition of each respiratory disease. A limit is that cardiovascular events were self-reported. However, a previous study from the general population showed a good sensitivity and specificity for self-reported diagnosis of cardiovascular events [24].

We conclude that a better understanding of the relationship between respiratory and cardiovascular diseases could have important clinical implications. First of all, CB, which is often considered as a minor symptom, has to be viewed as a status possibly evolving not only to irreversible airway obstruction [3] but also to cardiovascular damage. Secondly, there are possible consequences for disease management, such as screening, prevention and early treatment of cardiovascular diseases and risk factors in patients with chronic bronchitis, even in absence of irreversible airflow obstruction. In turn, in patients with cardiovascular diseases caution should be adopted about the use of drugs that could negatively interfere with the respiratory system (e.g. angiotensin-converting enzyme inhibitors potentially inducing cough).

In our study, cases of CB may also suffer from asthma and rhinitis. Thus our data suggest the crucial weight of cough and phlegm in driving the association between respiratory and cardiovascular risk factors and diseases. As a matter of fact, the strength of the associations was lower among subjects with asthma or rhinitis who did not complain of CB. This attention to CB may have also a strong preventive consequence, since the disease is generally present even without a clinically significant airway derangement. A similar consideration is also of importance for rhinitis, taking into account its association with peripheral arterial disease.

Finally, although the design of our study does not allow definitive conclusions, we are tempted to speculate that some cardiovascular risk factors, such as sedentariness, hypertension and dyslipidaemia, might also be involved in the development of respiratory diseases.

Supporting information

S1 Dataset. Minimal anonymised data set to replicate the analyses.
(CSV)

Author Contributions

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Original Contribution

Regular Physical Activity Levels and Incidence of Restrictive Spirometry Pattern: A Longitudinal Analysis of 2 Population-Based Cohorts

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We estimated the association between regular physical activity and the incidence of restrictive spirometry pattern. Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), and physical activity were assessed in 2 population-based European cohorts (European Community Respiratory Health Survey: $n = 2{,}757$, aged 39–67 years; and Swiss Study on Air Pollution and Lung and Heart Diseases in Adults: $n = 2{,}610$, aged 36–82 years) first in 2000–2002 and again approximately 10 years later (2010–2013). Subjects with restrictive or obstructive spirometry pattern at baseline were excluded. We assessed the association of being active at baseline (defined as being physically active at least 2–3 times/week for $\geq$1 hour) with restrictive spirometry pattern at follow-up (defined as a postbronchodilation FEV_1/FVC ratio of at least the lower limit of normal and FVC of $<$80% predicted) using modified Poisson regression, adjusting for relevant confounders. After 10 years of follow-up, 3.3% of participants had developed restrictive spirometry pattern. Being physically active was associated with a lower risk of developing this phenotype (relative risk = 0.76, 95% confidence interval: 0.59, 0.98). This association was stronger among those who were overweight and obese than among those of normal weight (*P* for interaction = 0.06). In 2 large European studies, adults practicing regular physical activity were at lower risk of developing restrictive spirometry pattern over 10 years.

BMI; FVC; physical activity; restrictive spirometry; spirometry

Abbreviations: BMI, body mass index; CI, confidence interval; ECRHS, European Community Respiratory Health Survey; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; SAPALDIA, Swiss Study on Air Pollution and Lung and Heart Diseases in Adults; RR, relative risk.

Restrictive spirometry pattern is an underrecognized disorder associated with high morbidity and mortality (1–3). It is characterized by low forced vital capacity (FVC), with a preserved ratio of forced expiratory volume in the first second (FEV_1) to FVC. The prevalence of restrictive spirometry pattern varies according to the definition used, ranging between 5% and 10% in the United States (3, 4) and Europe (5, 6). The determinants of restrictive spirometry pattern remain largely unknown.

We hypothesize that regular physical activity could be related to restrictive spirometry pattern incidence given that physical activity has been consistently associated with higher lung-function levels (7), and individuals with restrictive spirometry pattern were found to have low levels

of physical activity in a cross-sectional multicenter analysis (8). The important health benefits of regular physical activity and the modifiability of this behavior, combined with the largely unknown determinants of restrictive pattern incidence, makes this a highly relevant public health research question.

This study aimed to determine whether regular physical activity was associated with the development of restrictive spirometry pattern using data from 2 population-based adult cohorts followed for 10 years.

METHODS

Study design

We used longitudinal data collected from the European Community Respiratory Health Survey (ECRHS), involving 46 centers in 24 countries, and the Swiss Study on Air Pollution and Lung and Heart Diseases in Adults (SAPALDIA), involving 8 centers in Switzerland. Both studies followed a very similar protocol, using highly comparable questionnaires and procedures (9, 10). The present analysis used data collected during ECRHS II and SAPALDIA 2 (around 2000–2002) and approximately 10 years later during ECRHS III and SAPALDIA 3, hereafter referred to as the baseline and follow-up examinations. Subjects lost to follow-up (25% of the baseline sample) were on average older and more likely to have a higher body mass index (BMI), to report asthma, and to be a smoker than subjects who were included, as previously published (11).

All participants with obstructive spirometry pattern at baseline or follow-up and participants with restrictive spirometry pattern at baseline (definitions below) were excluded. Written informed consent was obtained from all participants and the appropriate institutional ethics committees in each participating center approved the study.

Variables

Lung function was assessed by spirometry according to American Thoracic Society recommendations (12). Details of the spirometers used in each center are provided elsewhere (7). At baseline, prebronchodilation measurements were performed. At follow-up, spirometry was performed both before and after bronchodilation (15 minutes after administering two 100-μg puffs of salbutamol using a spacer). We derived the percent-predicted value for FVC and the lower-limit of normal for the FEV_1/FVC ratio using study-specific equations (6, 13). A restrictive spirometry pattern was defined as having a postbronchodilation FEV_1/FVC of at least the lower limit of normal and a FVC of <80% predicted, as done previously with these population-based data (8) to maximize sample size (5). An obstructive spirometry pattern was defined as FEV_1/FVC below the lower limit of normal.

Subjects with neither a restrictive nor obstructive spirometry pattern were defined as having normal spirometry.

Physical activity was assessed by questionnaire and reported at baseline. Subjects were categorized as being regularly active if they reported usual practice of vigorous physical activity at least 2–3 times a week and with a duration of about 1 hour or more, as previously (7, 8, 14). All other participants were defined as nonactive.

Information on baseline characteristics (age, sex, education, smoking status, and passive smoking), current (within the previous 12 months) respiratory symptoms (asthma attack, wheezing, woken with tight chest, woken by attack of shortness of breath, woken by attack of coughing, and avoiding exercise because of breathing problems), chronic respiratory symptoms (ever asthma, chronic bronchitis, and chronic cough), and diagnosed chronic conditions (diabetes, depression, stroke, and hypertension) of the participants was collected using questionnaires. BMI was derived using height and weight measured at baseline and follow-up and classified as normal (<25.0), overweight (25.0–29.9), or obese (≥30.0) according to World Health Organization classifications (15).

Statistical analysis

Given that ECRHS and SAPALDIA share a very similar protocol, their recruitment was conducted at similar time-periods, and restrictive spirometry pattern incidence was comparable between studies in both sexes, data were pooled to maximize statistical power. Pooling of data was further supported by the lack of differences in the obtained estimates and the absence of evidence of heterogeneity when results were stratified by study and meta-analyzed (data not shown).

We assessed the association between physical activity reported at baseline and new onset of restrictive spirometry pattern using modified Poisson regression, adjusting for age, sex, smoking status, potential confounders that were significantly associated with both physical activity and restrictive spirometry pattern (education, BMI, passive smoke exposure, current and chronic respiratory symptoms, and chronic conditions), and baseline FVC levels. Statistical significance was set at $P < 0.05$. Center was included as a random effect. Analyses stratified by sex, smoking, study, and BMI were performed to detect possible effect modification.

The following sensitivity analyses were performed to assess the robustness of the results to assumptions about definitions, confounding, and models: 1) Global Lung Function Initiative equations (16) were used to define restrictive spirometry pattern; 2) an additional adjustment for BMI change between baseline and follow-up was made; 3) asthmatics and 4) those who reported avoiding physical activity because of respiratory symptoms were excluded in separate analyses; and 5) lung function at first examination was removed as an adjustment variable. All analyses were done using Stata, version 14 (StataCorp LP, College Station, Texas).

RESULTS

A total of 5,293 participants (2,714 in ECRHS and 2,579 in SAPALDIA) were available after excluding subjects with a restrictive spirometry pattern at baseline (ECRHS, $n = 166$; SAPALDIA, $n = 121$) and obstructive spirometry at any examination (ECRHS, $n = 422$; SAPALDIA,

Table 1. Participant Characteristics at Baseline According to Spirometry Pattern at Follow-up, European Community Respiratory Health Survey and Swiss Study on Air Pollution and Lung and Heart Diseases in Adults, 2001–2011

Characteristic	Spirometry at Follow-up						
	Normal			Restrictive			
	No.	%	Mean (SD)	No.	%	Mean (SD)	*P* Value[a]
Female sex	2,585	50.5		100	57.8		0.058
Age, years			46.57 (9.70)			47.12 (10.38)	0.494
BMI[b]							<0.001
Underweight	59	1.2		3	1.7		
Normal	2,555	50.0		59	34.1		
Overweight	1,876	36.7		73	42.2		
Obese	615	12.0		38	22.0		
BMI change[c]			1.08 (2.13)			2.12 (2.96)	<0.001
Smoking status							0.328
Never smoker	2,356	46.2		76	43.9		
Former smoker	1,615	31.7		50	28.9		
Smoker	1,146	22.4		47	27.2		
Pack-years[d]	0.25 (0, 13)			0.15 (0, 17)			0.464
Exposed to passive smoking, previous 12 months	1,494	29.3		62	35.8		0.062
Education							0.131
Low	221	8.5		14	13.9		
Medium	860	33.0		28	27.7		
High	1,526	58.5		59	58.4		
Respiratory symptoms							
Had an attack of asthma, previous 12 months	99	1.9		9	5.2		0. 003
Wheezing/whistling, previous 12 months	696	13.6		40	23.1		<0.001
Woken with tight chest, previous 12 months	541	10.6		33	19.1		<0.001
Woken by attack of SOB, previous 12 months	234	4.6		10	5.8		0.458
Woken by attack of coughing, previous 12 months	1,287	25.2		51	29.5		0.198
Chronic cough[e]	237	4.6		14	8.1		0.036
Avoided vigorous exercise, previous 12 months[f]	78	1.5		6	3.5		0.043
Ever had the following conditions							
Doctor-diagnosed asthma	384	7.5		19	11.0		0.090
Chronic bronchitis[g]	658	12.9		26	15.0		0.403
Hypertension	578	13.6		34	25.8		<0.001
Heart Disease	221	5.2		10	7.5		0.243
Depression	334	7.8		12	9.0		0.629
Diabetes	158	3.7		6	4.5		0.640
Cancer	209	4.9		6	4.5		0.825
Stroke	113	2.6		3	2.2		0.771

Table continues

Table 1. Continued

Characteristic	Spirometry at Follow-up						
	Normal			Restrictive			
	No.	%	Mean (SD)	No.	%	Mean (SD)	*P* Value[a]
Physically active	1,860	36.3		45	26.0		0.005
FEV_1, % predicted			101.93 (10.77)			88.20 (6.07)	<0.001
FVC, % predicted			103.37 (11.43)			89.93 (7.98)	<0.001
FEV_1/FVC			0.79 (0.06)			0.80 (0.06)	0.141
Study							0.057
ECRHS	2,613	51.0		101	58.4		
SAPALDIA	2,507	49.0		72	41.6		

Abbreviations: BMI, body mass index; ECRHS, European Community Respiratory Health Survey; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; SAPALDIA, Swiss Study on Air Pollution and Lung and Heart Diseases in Adults; SD, standard deviation; SOB, shortness of breath.

[a] *P* from χ^2 test (categorical) and analysis of variance (continuous).

[b] BMI calculated as weight (kg)/height $(m)^2$ and classified as normal (<25.0), overweight (25.0–29.9), or obese (≥30.0) according to World Health Organization classifications (15).

[c] BMI at follow-up − BMI at baseline.

[d] Values are expressed as median (25th, 75th percentile).

[e] Cough during the day or at night on most days for at least 3 months.

[f] Avoided taking vigorous exercise because of breathing problems.

[g] Cough and phlegm during the day or at night on most days for at least 3 months.

$n = 612$). At follow-up, 173 (3.3%) subjects had developed a restrictive spirometry pattern.

Table 1 shows the participant characteristics at baseline according to spirometry pattern at follow-up. Compared with participants with normal spirometry, those with new-onset restrictive spirometry pattern were more overweight or obese at baseline ($P < 0.001$); had a higher prevalence of respiratory symptoms such as chronic cough, wheezing, and waking with a tight chest, as well as hypertension; and had lower levels of FEV_1 and FVC (but not FEV_1/FVC ratio). Baseline characteristics according to physical activity are available in Web Table 1.

Being physically active at baseline was associated with a lower risk of having a restrictive spirometry pattern at follow-up (crude relative risk (RR) = 0.60, 95% confidence interval (CI): 0.46, 0.79, and adjusted RR = 0.76, 95% CI: 0.59, 0.98) (Figure 1).

There was no evidence of effect modification by smoking, sex, or study (Figure 1). However, analyses stratified by BMI groups suggested that the association between being active and new onset of restrictive spirometry pattern might be present only in those who were overweight (RR = 0.43, 95% CI: 0.24, 0.78) or obese (RR = 0.46, 95% CI: 0.17, 1.25). No association was found for normal-weight subjects (RR = 1.19, 95%CI: 0.69, 2.04). The *P* for interaction between overweight/obese versus normal weight and being active was 0.06. No substantial differences in the estimates were observed for any of the sensitivity analyses (Figure 2). However, the strength of the association was higher after removing FVC levels at baseline from the model and lower after additionally adjusting for change in BMI between the 2 examinations.

DISCUSSION

Our study reveals, we believe for the first time, an association of regular physical activity with a lower risk of developing restrictive spirometry pattern over 10 years in 2 longitudinal, European, multicenter cohorts. This association remained after adjusting for baseline lung function and relevant confounders, such as active and passive smoking and the presence of respiratory symptoms.

Two previous cross-sectional studies have reported similar associations (8, 17). Although the temporality of the observed associations could not be explored, these previous studies suggested that restrictive spirometry pattern could reduce physical activity practice. Because our interest was in the association between physical activity and further restrictive spirometry pattern, we excluded all restrictive spirometry pattern cases at baseline and adjusted our analysis for baseline FVC to reduce any potential confounding of baseline lung function on physical activity (i.e., reverse causation).

Physical activity has been consistently associated with FEV_1 and FVC levels, and less so with their decline (7, 18). We hypothesize that the relationship between physical activity and lung function could take on different clinical expressions (i.e., obstructive or restrictive spirometry pattern) depending on the individual distribution of health determinants, which could result in different associations

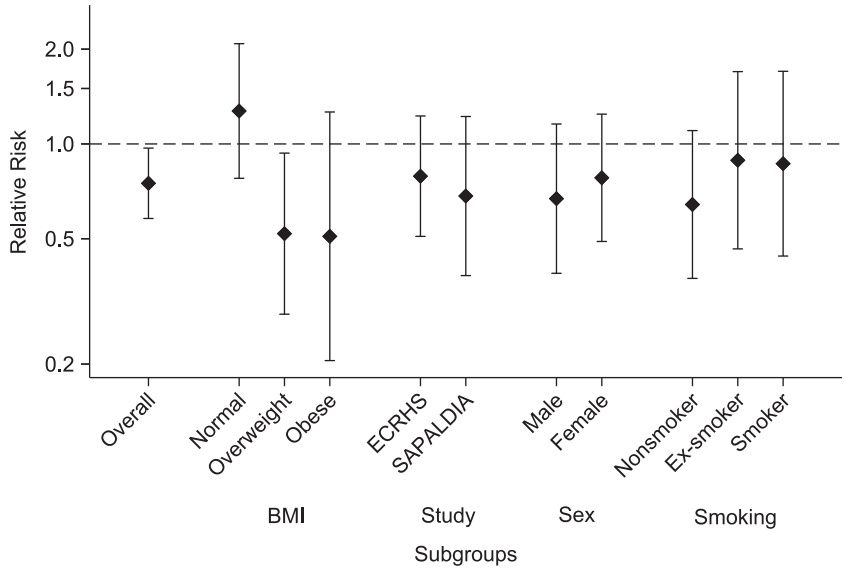

Figure 1. Relative risk of restrictive spirometry pattern incidence (active vs. nonactive), European Community Respiratory Health Survey (ECRHS) and Swiss Study on Air Pollution and Lung and Heart Diseases in Adults (SAPALDIA), 2001–2011. Overall, and stratified by body mass index, study, sex, and smoking status. Relative risk from modified Poisson regression, adjusted for age, sex, body mass index (BMI), chronic cough, woken by tight chest, avoiding physical activity because of respiratory symptoms, and forced vital capacity at baseline. Center was included as random effect.

between physical activity and FEV_1 and FVC. Supporting this hypothesis, physical activity appears to consistently protect against developing obstructive pattern among active smokers only (7, 18). Given that we did not observe any effect modification by smoking, we suggest that mechanisms other than those involved in the obstructive pattern might

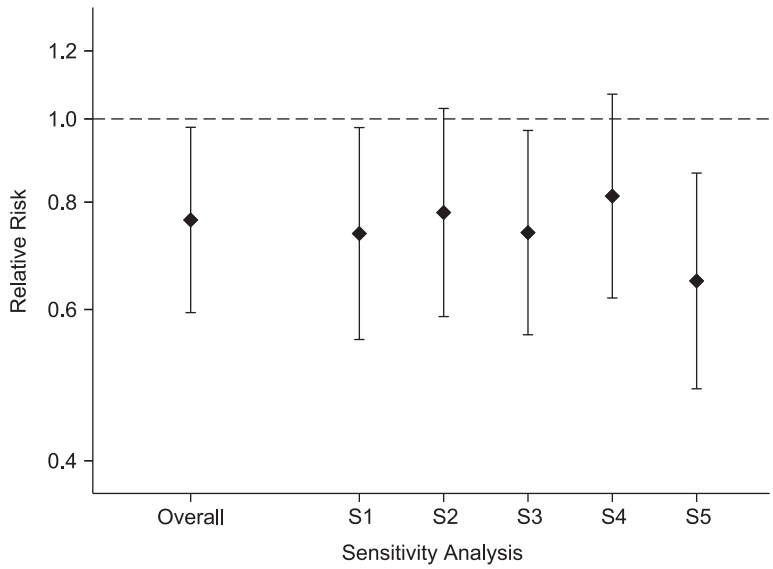

Figure 2. Sensitivity analyses of relative risk of restrictive spirometry pattern incidence (active vs. nonactive), European Community Respiratory Health Survey and Swiss Study on Air Pollution and Lung and Heart Diseases in Adults, 2001–2011. Relative risk from modified Poisson regression, adjusted for age, sex, body mass index, chronic cough, woken by tight chest, avoiding physical activity because of respiratory symptoms, and forced vital capacity at baseline. Center was included as random effect. S1: Global Lung Function Initiative equations (16) were used instead of study-specific equations to define spirometry patterns. S2: Additionally adjusted for change in body mass index between baseline and follow-up. S3: Excluding participants with asthma. S4: Excluding subjects who avoided physical activity because of their respiratory symptoms. S5: No adjustment for forced vital capacity levels at baseline.

underlie the protective association between physical activity and the development of restrictive spirometry pattern.

Among the possible causes, restrictive spirometry pattern can generally be attributed to abnormalities either in the lung itself (e.g., fibrosis) or outside of it (e.g., obesity, chest wall deformities) that impair one's ability to fully inflate the lungs. Thus, physical activity might exert its preventive action in relation to restrictive spirometry pattern development through mechanisms playing a role both inside and outside the lung. One hypothesis is that physical activity prevents weight gain in the aging population, whose lung function is in the declining phase. Indeed, weight gain (BMI increase) has been associated with accelerated FVC decline over 10 years in adults (19), which could result in a higher risk of incident restrictive spirometry pattern. This hypothesis is not fully supported by our study because the association between physical activity and the incidence of restrictive spirometry pattern remained after adjustment for BMI changes during follow-up. However, we acknowledge that BMI might not appropriately reflect fat mass and that fat and lean mass have different impacts on lung function (20).

Another possible mechanism is that regular physical activity prevents systemic inflammation. Indeed physical activity has antiinflammatory effects (21), and systemic inflammation is associated with lower lung function (22). Furthermore, subjects with restrictive spirometry pattern have elevated levels of fibrinogen and C-reactive protein (23, 24), 2 markers of inflammation. In our study, associations were strongest among those who were overweight or obese (who have higher systemic inflammation (25)), possibly suggesting that the antiinflammatory effects of physical activity are most important in this high-risk population. However, we did not observe a similar association among smokers, another group with a high inflammatory burden. Further research is warranted to confirm or refute the systemic inflammation pathway as a viable mechanism to explain our findings.

A limitation of this study is that physical activity was assessed using questionnaires, which are likely subject to nondifferential misclassification. Second, some subjects were lost to follow-up, and they exhibited generally worse health at baseline, which might have introduced some bias in our estimates. Third, residual confounding is possible but unlikely because most known relevant confounders were accounted for and unknown confounders are unlikely to account for all of the observed estimate. Fourth, a single measurement of physical activity 10 years before incidence might lack precision and most likely biases our estimates toward the null. Fifth, we were unable to test whether BMI is a mediating factor due to the small number of observed, incident cases of restrictive spirometry pattern. Finally, the exclusion of subjects with obstructive spirometry pattern, done to facilitate the interpretation of the results, might have lowered the statistical power because some subjects develop a mixed pattern (i.e., combination of obstructive and restrictive spirometry pattern).

Although we adjusted for baseline lung function and respiratory symptoms, we cannot exclude the possibility that reverse causation exists to some extent—for example, due to a potential association between early restrictive disease and exercise limitation leading to physical inactivity.

The strengths of the present study are its longitudinal design and the combination of 2 large population-based cohorts with long follow-ups. Although the wide geographical distribution across Europe and the randomly selected participants make our results generalizable to diverse population subgroups, our findings should be replicated in other settings. Furthermore, high-quality lung-function data were available. In particular, restrictive spirometry pattern was defined according to postbronchodilation lung-function measurements, which are subject to less misclassification than prebronchodilation measurements (5). The adjustment by baseline FVC to reduce reverse causation is a further strength. However, this strategy could have underestimated the magnitude of the association between physical activity and the development of restrictive spirometry pattern (where lower FVC levels are associated with faster decline, the so-called horse-racing effect (26)).

From a public health perspective, our study carries an important message: Restrictive spirometry pattern might need to be added to the list of disorders associated with low physical activity levels. Because having a restrictive spirometry pattern is associated with poor quality of life (6) and mortality (3), if regular physical activity can truly prevent its occurrence, it might also prevent hospitalizations and deaths.

In conclusion, in a large study across Europe, being physically active was associated with a reduced risk of developing restrictive spirometry pattern over 10 years. This result reinforces the importance of promoting regular practice of physical activity in the general population to improve general health and prevent disorders later in life.

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Cumulative occupational exposures and lung function decline in two large general population cohorts

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Abstract

Rationale: Few longitudinal studies have assessed the relationship between occupational exposures and lung function decline in the general population, with sufficiently long follow-up.

Objectives: Our objective was to examine this potential association in two large cohorts (ECRHS and SAPALDIA).

Methods: General population samples aged 18 to 62 were randomly selected in 1991-1993, and followed up approximately 10 and 20 years later. Spirometry (without bronchodilation) was performed at each visit. Coded complete job histories during follow-up visits were linked to a Job-Exposure Matrix, generating cumulative exposure estimates for 12 occupational exposures. FEV_1 and FVC were jointly modelled in linear mixed-effects models, fitted in a Bayesian framework, taking into account age and smoking.

Main results: A total of 40,024 lung function measurements from 17,833 study participants were analyzed. We found accelerated declines in FEV_1 and the FEV_1/FVC ratio for exposure to biological dust, mineral dust and metals (FEV_1 -15.1ml, -14.4ml and -18.7ml respectively, and FEV_1/FVC -0.52%, -0.43% and -0.36% respectively, per 25 intensity-years of exposure). These declines were comparable in magnitude to those associated with long-term smoking. No effect modification by sex or smoking status was identified. Findings were similar between the ECRHS and SAPALDIA cohorts.

Conclusions: Our results greatly strengthen the evidence base implicating occupation, independent of smoking, as a risk factor for lung function decline and by inference for COPD. This highlights the need to prevent or control these exposures in the workplace.

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Introduction

Chronic Obstructive Pulmonary Disease (COPD) is an important cause of population morbidity and mortality, characterized by a low level of lung function and persistent airflow limitation (1). The most well-recognized risk factor is tobacco smoking, which is associated with the majority of COPD cases. Other environmental risk factors are also implicated in the pathogenesis of COPD (2), including occupational exposures (3). The European Community Respiratory Health Survey (ECRHS) has recently shown a higher incidence of COPD in workers exposed to biological dusts, gases, fumes and pesticides, with a combined population attributable fraction of 21% (4).

Lung function declines naturally with age, but an accelerated decline is a primary, though not obligate, cause of COPD (5), as well as a long-term feature of asthma (6). Despite a large number of studies, both population- and industry based, demonstrating an association of asthma and COPD with occupational exposures (3, 7, 8), relatively few population-based longitudinal studies have examined the relationship between lung function decline and occupational exposures as estimated by a Job-Exposure Matrix (JEM) (9–11). A previous analysis from the first 10-year follow-up of the European Community Respiratory Health Survey (ECRHS) did not show a steeper decline in lung function in people exposed to vapors, gases, dusts or fumes (9); the cohort however was fairly young then (30-55 years old at the time), and the follow-up time may have been too short to detect an association.

Therefore our aim was to examine the association between occupational exposures and the rate of lung function decline, given its relevance to COPD risk. To do so, we combined two large prospective cohorts participating in the ALEC project (www.alecstudy.org): the ECRHS (12) and the Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults (SAPALDIA) (13). Both cohorts have accumulated two decades of follow-up, with more

participants over 50 years of age, thus allowing better and more precise estimates of the determinants of lung function decline. In addition, we examined whether the association between occupation and lung function decline is different between men and women, and between smokers and non-smokers.

Methods

The ECRHS is a multicentre longitudinal study initiated in 1991–1993 that enrolled random general population samples aged 20 to 44 years in 55 centres from 23 countries (12). Participants at baseline (ECRHS I) completed a detailed questionnaire via face-to-face interview and underwent a clinical examination, spirometry and other measurements. They were followed again between 1998 and 2002 (ECRHS II), and a second time between 2010 and 2012 (ECRHS III). SAPALDIA is also a multicentre longitudinal study with objectives, methods and protocols very similar to those of the ECRHS, that was initiated in 1991 (SAPALDIA 1) and enrolled a random general population sample aged 18 to 62 years from eight regions in Switzerland (13). SAPALDIA participants were also followed up in 2001 and 2011 (SAPALDIA 2 and 3). A flowchart of study participants from both cohorts can be found in the online supplement (Supplemental Figure 1).

During both follow-ups, participants in both cohorts were asked to provide a detailed list of their occupations and industries from all jobs held since the last study visit; these were recorded in free text and subsequently coded in the International Classification of Occupations-88 (ISCO-88) standard by trained local coders, who were blind to participant status. Ethical approval for each centre was obtained from their respective competent bodies, and written informed consent was obtained from all participants.

The population for this analysis includes all participants who completed spirometry at baseline (ECRHS I / SAPALDIA 1) and were followed up at least once (at ECRHS II / SAPALDIA 2 and/or ECRHS III / SAPALDIA 3). All spirometries were performed without bronchodilation and according to the ATS/ERS standards for reproducibility, utilizing the maximum Forced Volume Capacity (FVC) and Forced Expiratory Volume in 1 second (FEV_1) per participant. Occupational exposures were determined by linking the participants ISCO-88 coded occupations to the semi-quantitative ALOHA(+) JEM (10). This JEM assigns, for every ISCO-88 job code, three grades of exposure (none, low, high) to twelve agents (biological dusts, mineral dusts, gases/fumes, herbicides, insecticides, fungicides, aromatic solvents, chlorinated solvents, other solvents, and metals) including two composites of the above (All pesticides, and Vapors/Gases/Dusts/Fumes, VGDF). For each participant a cumulative exposure to each agent in intensity-years was calculated, by multiplying the duration of each job with the intensity of exposure (0 for none, 1 for low, and 4 for high); these intensities reflect the lognormal distribution of occupational exposure concentrations when calculating cumulative exposures.

Covariates used for adjustment included at each visit age, sex, height (including its square, to allow for non-linear associations), current asthma, current smoking, lifetime smoking pack-years, Socioeconomic Status (SES) and early life disadvantage score; the latter is a composite variable that includes maternal smoking, maternal asthma, paternal asthma, childhood asthma (before age 10), and having a serious respiratory infection before age 5 (14). Current asthma was defined as a positive response to either of the following three questions: “have you had an attack of asthma in the last 12 months?”, “are you currently taking any medicines for asthma?” and “have you been woken by an attack of shortness of breath at any time in the last 12 months?”. SES was defined according to the participants' age of completion of formal

education, and classified into three categories: high (>19 years), middle (16-19 years), low (<16 years).

Associations between cumulative occupational exposures and lung function (FEV_1, FVC and the FEV_1/FVC ratio) were assessed using linear mixed-effects models, providing the mean change in lung function per intensity-year of exposure. FEV_1 and FVC were jointly modelled, and all models included participant-level random intercepts and slopes, taking account of the correlations both between random intercepts and slopes as well as between FEV_1 and FVC. For each ALOHA(+) exposure agent we fitted two joint models, one using absolute FEV_1 and FVC ("linear model") and one using their logarithms as the outcome ("log-linear model"); from the latter we calculated the effects of exposures on the FEV_1/FVC ratio, as the difference between model parameters for log(FEV_1) and log(FVC). In all instances, the comparison was between exposed and unexposed participants to the particular ALOHA(+) agent, not against participants without any occupational exposure.

The models were fitted in a Bayesian framework with the JAGS software, setting non-informative priors for all parameters, and using 4 chains and 20,000 iterations per chain, discarding the first 2,000 as burn-in, and with a thinning interval of 5. Convergence was checked by visual inspection of the MCMC traceplots and by the Gelman-Rubin statistic. Furthermore, all models included a fully Bayesian imputation sub-model for handling item (covariate) missingness, with hyperparameters set to non-informative priors (see online supplement for details). Uncertainty for the fixed effects was expressed with 95% Credible Intervals (95% CrI).

In addition to the unstratified models, we fitted another set of models with added interaction terms for sex, and smoking status (ever/never smoker), thereby calculating stratified estimates for the effect of occupational exposures in men and women, and in ever smokers and never

smokers. As a criterion to determine the presence of interaction (effect modification) we used the posterior probability distribution of the interaction term, at least 95% of which should lie above or below zero. We also fitted all models separately to the data from each cohort (ECRHS and SAPALDIA), as a sensitivity analysis.

All analyses were performed with the R statistical environment, version 3.6.0 (15).

Results

Table 1 highlights the characteristics of the study population. We analyzed a total of 40,024 lung function measurements from 17,833 study participants across 38 ECRHS and SAPALDIA centres who completed at least one follow-up visit after baseline, with a mean follow-up duration of 16.3 years (range 4.3–22.6 years). Of these participants, 10,803 (60.6%) completed both visits over a mean duration of 19.6 years (range 15.7–22.6 years). 5,793 (32.5%) participants completed the initial and first follow-up visits only, while 1,237 (6.9%) completed the initial and second follow-up visits only. Slightly less than half of our sample had never smoked, and about a third were current smokers at baseline, dropping almost by half to 18.3% at the second follow-up. A little less than half of all participants had been occupationally exposed to VGDF at some point during follow-up (39.8%), whereas fewer had been exposed to solvents (24.2%), metals (9.3%) or pesticides (3.3%). Men were overall more likely than women to be occupationally exposed to most agents, with the exception of biological dust (Table 2). In addition, many exposures showed substantial overlap with each other (Figure 1). A list of the most common jobs by exposure category can be found online (Supplemental Table 1).

Lifetime smoking pack-years were missing in 7.8% of all observations, and had to be imputed in our models as described in the "Methods" section; also current smoking status was missing in 2.9%, current asthma in 0.5% and SES in 1.1% of all observations. Lung function in the study population naturally declined with advancing age across both follow-ups, and our Bayesian mixed-effects model was reliable in describing both mean lung function by age as well as the variability around the mean (Figure 2).

Table 3 summarizes the main results from our analysis, i.e. the effect of 25 intensity-years of occupational exposure to each ALOHA(+) agent on the three lung function parameters (FEV_1, FVC and the FEV_1/FVC ratio), both overall and stratified by sex and smoking status. A negative sign indicates reduced lung function compare to no occupational exposure to the respective agent given the same age, sex and other covariates, i.e. an accelerated lung function decline, whereas a positive number indicates a slower lung function decline. As the numbers are very small, the effect per 25 intensity-years is presented rather than per one intensity-year of exposure.

A decreased FEV_1/FVC ratio was observed for biological dust and mineral dust exposure (-0.52% and -0.43% respectively, Table 3), which was purely attributed to a FEV_1 decline (-15.08 ml and -14.42 ml respectively) with no change in FVC. A significant decline in FEV_1 only was also observed for metals exposure (-18.73 ml, 95% CrI -34.41 to -2.60 ml), as well as a lower FEV_1/FVC ratio for the composite VGDF exposure (-0.34%, 95% CrI -0.56 to -0.12%). On the other hand, smaller lung function declines were seen for gases & fumes (-7.35 ml in FEV_1 and -0.24% in FEV_1/FVC), and also for pesticides, especially in terms of FEV_1, although the results were very imprecise, with a wider 95% CrI than other exposures (Table 3). Among solvents, only exposure to chlorinated solvents was weakly associated with both lower FEV_1 and lower FVC (-16.98 ml and -14.59 ml). For comparison, as estimated in the

model, 25 pack-years of smoking reduced FEV1 by an additional -11.07 ml (95% CrI -22.27 to 2.49 ml), FVC by -14.83 ml (95% CrI -32.29 to -0.55 ml) and FEV1/FVC by -0.21% (95% CrI -0.44 to 0.03%). Also, current asthma was associated with a FEV1 that was -80.28 ml lower (95% CrI -92.72 to -67.71 ml), a FVC lower by -13.60 ml (95% CrI -27.44 to -0.05 ml) and a FEV1/FVC -2.16% lower (95% CrI -2.52 to -1.81%).

No effect modification by sex was detected for any occupational exposure and any lung function parameter, with one exception: women exposed to aromatic solvents had a slower FVC decline than unexposed women (+86.87 ml, 95% CrI 13.80 to 161.25 ml), whereas no significant difference was found among men (-7.84 ml, 95% CrI -34.96 to 20.18 ml). In addition, no effect modification by smoking status was detected, although in most cases the effects of occupational exposures in ever smokers tended to be slightly lower than in never smokers (Table 3). In the sensitivity analysis, results were similar between the two cohorts and showed wide overlap; pesticide exposure resulted in greater FEV_1 decline among ECRHS participants, while solvents resulted in slightly greater declines among SAPALDIA participants (Supplemental Table 2).

Discussion

In this pooled analysis from two large longitudinal cohorts we showed that certain groups of occupational exposures, namely biological dust, mineral dust and metals exposure, were prospectively associated with accelerated lung function decline, specifically with respect to FEV1 and the FEV1/FVC ratio. This loss of lung function is comparable to that associated with smoking, highlighting the importance of these occupational exposures in respiratory health. Our study provides significant new evidence on the topic, as few such studies have

examined longitudinal lung function decline and in a general population setting (10, 11, 16–18). In comparison to industry-based studies, general population cohorts can provide more generalizable information by including all types of exposures across all industries, and by adjusting for socioeconomic status and other covariates. Previous analyses from both ECRHS and SAPALDIA have shown an increased asthma risk for certain occupational exposures (19), an increased incidence of COPD in participants occupationally exposed to biological dust and mineral dust (4, 20), as well as an increased incidence of chronic cough and chronic phlegm symptoms in those exposed to mineral dust and metals (21). Our current analysis corroborates previous findings for biological dust in a much larger study population with additional follow-up. It also indicates that mineral dust and metals exposure are associated also with lung function decline and not only with chronic bronchitis symptoms. The latter result is in agreement with recent findings from the smaller Tasmanian longitudinal Health Study (TAHS) cohort (22), as well as with a systematic review of exposure to welding fumes and lung function decline (23).

A striking finding is the very small absolute effect sizes observed, only a few millilitres (or a fraction of a percentage point) of lung function decline after 25 intensity-years of exposure, which for many workers may represent their entire lifetime exposure. At first glance such reductions could not plausibly account for an increased COPD risk as seen in previous analyses (4, 8, 20); however, these are average subject-specific effects over an exposure group, and small average reductions can mask a larger effect for certain individuals inside the group (24), especially if one also considers the sizeable individual variation in lung function (Figure 2). In addition, the comparator is not a fully occupationally unexposed group, but rather participants unexposed to the agent under consideration (who may have other occupational exposures). Furthermore, non-differential misclassification is the main limitation

in using JEMs in population-based occupational epidemiology studies, mostly stemming from the variability in job tasks and actual exposures between workers belonging in the same JEM exposure category (25). Grouping exposures in a JEM does not in itself bias regression coefficients towards the null, but rather results in imprecision due to Berkson-type error (26); nevertheless, regression dilution bias can be introduced if the estimated group mean is different than the true group mean (27). Intra-individual variability in exposure over time is another consideration, which is particularly relevant when calculating a cumulative exposure estimate based on job duration and a JEM classification, in this case over a long period of 20 years (28). All these factors mean that the small average declines observed in our analysis should not be used for prediction, as they are unlikely to represent the actual magnitude of the effect of these exposures on lung function, in any particular individual. For the same reasons, our results cannot definitively rule out an effect of occupational solvent exposure on lung function as recently observed in the TAHS cohort (22), nor an effect of pesticide exposure (4, 10). Especially as regards pesticides, the small percentage of exposed participants (less than 5% – Table 2) has substantially reduced precision, as reflected in the wider credible intervals of the lung function decline estimates.

Smoking is known to induce inflammation and impair the host defense mechanisms of the lung (29, 30), and has been reported to increase the adverse effect of occupational exposures on lung function decline and COPD risk (31–33). However, in our analyses we found little evidence of effect modification by smoking, after tightly adjusting both for current smoking status and cumulative pack-years. This indicates that all workers may be at risk of airway obstruction due to these occupational exposures, even though this may be more clinically significant for smokers, who represent the majority of COPD patients in most countries, and who already have lower levels of lung function and any additional loss or acceleration of

decline may push them over the diagnostic cutoff. Similarly, we found little difference between men and women on the effect of occupational exposures, even though the distribution of jobs per each exposure category was different between men and women. This may partially be explained by the lower proportion of occupationally exposed women in our cohorts, particularly for exposures like metals, pesticides and solvents. Future occupational epidemiology studies should try to recruit more women, in order to investigate effect modification by sex.

Strengths of the current study include its prospective population-based design and long follow-up of 20 years. Full job histories were collected for the study period and cumulative occupational exposures were calculated using a JEM instead of self-report; the latter could be vulnerable to recall bias, especially given the long follow-up involved. Lung function was modelled in great detail, using a mixed-effects model with random intercepts and slopes, and accounting for the correlation between FEV1 and FVC; joint modelling of both spirometric parameters is important, as we identified positive correlations not just between the participant-level random intercepts and slopes for FEV1 and for FVC, but also between the intercepts for FEV1 and FVC and the slopes for FEV1 and FVC (data not shown). In addition we controlled for multiple confounders, including socioeconomic status, current asthma and especially lifetime smoking pack-years, in order to minimize confounding by intensity of smoking. The sample size was very large, thanks to the pooling of two large cohorts, facilitating the detection of associations of very small magnitude.

On the other hand, there are certain limitations to our study. Spirometries were performed without bronchodilation, and the results should be interpreted accordingly. Accelerated lung function decline in and of itself does not equate with COPD risk; in particular, occupation can also be a risk factor for asthma, thus it is not possible to distinguish lung function decline due

to new-onset occupational asthma from that contributing to increased COPD incidence. The application of a JEM may ensure more objective exposure estimates, free from exposure-specific recall, but can introduce both imprecision due to Berkson-type error and some bias towards the null; therefore, despite the large sample size, it may not have been always sufficient to detect an association, e.g. for the effects of pesticides or to identify effect modification. For the same reason, we cannot disentangle the effect of multiple overlapping exposures, which would require a large number of small subgroup analyses. We could also not assess heterogeneity of the results across study centres or countries, since the (necessary) inclusion of participant-level random effects left almost no variance to be explained by study centre. Finally, residual confounding cannot be completely ruled out despite the detailed model adjustments, and may have affected the observed associations given their small magnitude. As this is an observational study, some selection and response bias also cannot be definitely ruled out.

In conclusion, long-term occupational exposure to biological dust, mineral dust and metals over two decades of follow-up was associated with an accelerated decline in FEV_1 and the FEV_1/FVC ratio, which would translate to an increased risk of airways obstruction and COPD. This decline was comparable to that associated with smoking, and similar between men and women as well as between smokers and non-smokers. These results strengthen the case for occupation as a modifiable risk factor for asthma and COPD, in agreement with previous studies. And they make the case for avoiding these exposures in the workplace, or controlling them with appropriate protective measures, in order to protect the respiratory health of workers.

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Figure Legends

Figure 1: Correlation matrix (Spearman's rho) between cumulative occupational exposures (intensity-years) in the study population, as estimated by the ALOHA(+) JEM. (N=17,833 ECRHS and SAPALDIA participants)

Figure 2: Lung function by age and sex in the study population, observed and model-predicted. (N=40,024 measurements in 17,833 ECRHS and SAPALDIA participants)

Footnote for Figure 2:

Height set to cohort mean by sex; smoking pack-years and cumulative VGDF exposure set to cohort mean by age and sex.

Table 1: Characteristics of study participants, by study wave. (N=17,833 ECRHS and SAPALDIA participants followed-up at least once)*

	ECRHS I / SAPALDIA 1	ECRHS II / SAPALDIA 2	ECRHS III / SAPALDIA 3
Number of participants	17833 (9765 / 8068)	16596 (8725 / 7871)	12040 (6013 / 6027)
% men	48.0 (48.0 / 47.9)	48.1 (48.1 / 48.0)	47.7 (47.9 / 47.5)
Mean age	37.2 (34.0 / 41.0)	47.2 (42.8 / 52.0)	56.9 (54.1 / 59.7)
% current asthma	6.4 (7.9 / 4.7)	7.9 (9.9 / 5.6)	7.6 (11.2 / 4.1)
% never smokers	46.0 (44.8 / 47.4)	45.9 (45.5 / 46.4)	48.5 (49.0 / 48.1)
% current smokers	33.3 (34.5 / 31.9)	27.7 (29.0 / 26.3)	18.3 (18.1 / 18.5)
Mean cumulative smoking pack-years	8.4 (7.2 / 10.0)	11.1 (9.7 / 12.7)	11.7 (10.9 / 12.3)
% of participants exposed			
Biological dust	-	22.0 (26.6 / 17.0)	26.7 (30.7 / 22.6)
Mineral Dust	-	17.6 (21.1 / 13.8)	20.9 (23.9 / 18.0)
Gases & fumes	-	31.7 (37.4 / 25.4)	37.4 (41.3 / 33.5)
Vapors, Gases, Dusts & Fumes	-	35.8 (41.9 / 29.2)	42.0 (46.1 / 37.9)
Herbicides	-	1.7 (1.5 / 1.9)	2.2 (1.8 / 2.6)
Insecticides	-	2.1 (2.3 / 1.9)	2.8 (2.9 / 2.6)
Fungicides	-	2.2 (2.4 / 2.0)	3.1 (3.4 / 2.8)
All pesticides	-	2.7 (3.2 / 2.1)	3.6 (4.3 / 3.0)
Aromatic solvents	-	11.4 (13.1 / 9.6)	13.5 (14.9 / 12.1)
Chlorinated solvents	-	9.1 (10.3 / 7.7)	10.9 (12.2 / 9.6)
Other solvents	-	19.4 (23.0 / 15.3)	23.6 (26.9 / 20.4)
Metals	-	7.9 (9.5 / 6.1)	9.7 (11.4 / 8.0)
Mean cumulative exposures since previous follow-up (intensity-years)			
Biological dust	-	2.2 (2.2 / 2.1)	4.1 (4.7 / 3.6)
Mineral Dust	-	2.3 (2.4 / 2.2)	4.1 (4.7 / 3.5)
Gases & fumes	-	3.6 (3.9 / 3.3)	6.6 (7.7 / 5.4)
Vapors, Gases, Dusts & Fumes	-	5.1 (5.3 / 4.8)	9.2 (10.6 / 7.9)
Herbicides	-	0.2 (0.1 / 0.3)	0.4 (0.3 / 0.6)
Insecticides	-	0.4 (0.3 / 0.6)	0.8 (0.6 / 1.1)
Fungicides	-	0.3 (0.3 / 0.4)	0.6 (0.6 / 0.6)
All pesticides	-	0.5 (0.4 / 0.6)	0.9 (0.7 / 1.1)
Aromatic solvents	-	1.0 (1.0 / 1.0)	1.7 (1.9 / 1.6)
Chlorinated solvents	-	1.2 (1.2 / 1.2)	1.9 (2.1 / 1.7)
Other solvents	-	1.7 (1.8 / 1.6)	3.2 (3.7 / 2.7)
Metals	-	1.2 (1.3 / 1.1)	2.0 (2.3 / 1.7)

* Data for the entire cohort (data for ECRHS / data for SAPALDIA)

Table 2: Proportion of participants with any occupational exposure during follow-up, stratified by sex. (N=17,833 ECRHS and SAPALDIA participants followed-up at least once)*

	% of men exposed	% of women exposed
Biological dust	22.2 (25.5 / 18.2)	27.7 (32.5 / 22.0)
Mineral Dust	28.8 (33.6 / 23.1)	11.8 (13.4 / 10.0)
Gases & fumes	42.7 (47.9 / 36.4)	29.0 (33.2 / 23.8)
Vapors, Gases, Dusts & Fumes	46.3 (51.8 / 39.7)	33.8 (38.2 / 28.4)
Herbicides	2.8 (2.5 / 3.1)	1.2 (1.0 / 1.5)
Insecticides	3.5 (3.8 / 3.1)	1.6 (1.6 / 1.5)
Fungicides	4.1 (4.7 / 3.4)	1.5 (1.4 / 1.6)
All pesticides	4.9 (5.9 / 3.7)	1.7 (1.8 / 1.6)
Aromatic solvents	21.5 (23.6 / 18.8)	5.0 (5.9 / 3.8)
Chlorinated solvents	16.8 (18.7 / 14.5)	4.5 (5.2 / 3.7)
Other solvents	25.5 (28.4 / 22.0)	18.9 (22.7 / 14.4)
Metals	17.3 (20.1 / 13.9)	1.8 (2.4 / 1.0)

* Data for the entire cohort (data for ECRHS / data for SAPALDIA)

Table 3: Effect of occupational exposures on lung function decline, per 25 intensity-years of exposure, compared to unexposed to the respective agent, overall and stratified by sex and smoking status. (N=17,833 ECRHS and SAPALDIA participants followed-up at least once)*

	FEV1 (ml)		FVC (ml)		FEV1FVC (%)	
Biological dust	**-15.08 (-28.66, -0.88)**		2.68 (-12.68, 18.28)		**-0.52 (-0.88, -0.16)**	
Never smokers, men / women	-3.23 (-26.67, 20.22)	-14.38 (-45.32, 15.59)	13.44 (-12.79, 39.41)	8.35 (-25.83, 43.27)	-0.28 (-0.95, 0.41)	-0.52 (-1.35, 0.36)
Ever smokers, men / women	-15.86 (-33.93, 2.09)	**-27.11 (-53.91, -1.19)**	-0.47 (-20.80, 19.98)	-5.59 (-35.49, 24.14)	**-0.52 (-1.02, -0.02)**	**-0.76 (-1.46, -0.04)**
Mineral Dust	**-14.42 (-26.41, -2.50)**		0.12 (-12.99, 13.10)		**-0.43 (-0.75, -0.10)**	
Never smokers, men / women	-9.38 (-28.04, 9.65)	5.55 (-29.53, 39.95)	10.96 (-10.24, 31.96)	15.54 (-22.24, 53.37)	**-0.56 (-1.10, -0.02)**	-0.31 (-1.23, 0.61)
Ever smokers, men / women	**-20.83 (-35.33, -6.25)**	-6.15 (-37.68, 24.57)	-7.12 (-23.55, 9.48)	-2.42 (-37.19, 33.52)	**-0.44 (-0.84, -0.03)**	-0.18 (-1.01, 0.65)
Gases & fumes	-7.35 (-17.99, 3.15)		3.46 (-8.66, 15.25)		-0.24 (-0.53, 0.04)	
Never smokers, men / women	4.22 (-12.83, 21.44)	-10.01 (-39.00, 19.03)	14.43 (-4.90, 33.41)	11.31 (-22.02, 43.81)	-0.14 (-0.64, 0.35)	-0.38 (-1.16, 0.43)
Ever smokers, men / women	-10.22 (-23.34, 3.14)	-24.37 (-51.63, 3.81)	-2.14 (-16.77, 12.32)	-5.52 (-36.24, 25.02)	-0.24 (-0.59, 0.11)	-0.48 (-1.24, 0.24)
Vapors, Gases, Dusts & Fumes	-7.42 (-15.93, 1.03)		6.02 (-3.04, 15.42)		**-0.34 (-0.56, -0.12)**	
Never smokers, men / women	-1.44 (-14.83, 11.91)	-6.21 (-28.65, 16.31)	11.89 (-2.73, 26.67)	14.92 (-10.55, 39.96)	-0.27 (-0.65, 0.11)	-0.47 (-1.06, 0.13)
Ever smokers, men / women	-9.45 (-19.62, 0.97)	-14.20 (-35.47, 7.03)	1.76 (-9.68, 13.41)	5.14 (-18.42, 28.40)	**-0.33 (-0.61, -0.06)**	-0.52 (-1.06, 0.01)
Herbicides	-14.69 (-49.54, 20.57)		-8.57 (-47.42, 29.56)		-0.34 (-1.28, 0.59)	
Never smokers, men / women	-19.16 (-85.70, 43.26)	35.35 (-51.37, 122.19)	-23.73 (-96.58, 48.69)	22.63 (-73.97, 120.70)	-0.28 (-2.17, 1.65)	0.55 (-1.85, 3.00)
Ever smokers, men / women	-33.13 (-77.76, 11.56)	21.78 (-46.93, 91.26)	-20.84 (-72.01, 28.90)	26.82 (-50.71, 104.30)	-0.67 (-1.88, 0.55)	0.12 (-1.71, 2.05)
Insecticides	-7.24 (-29.73, 15.04)		-2.43 (-27.68, 23.16)		-0.23 (-0.83, 0.38)	
Never smokers, men / women	-7.72 (-47.54, 32.36)	22.94 (-28.06, 74.16)	3.48 (-40.41, 46.91)	35.24 (-22.53, 92.33)	-0.50 (-1.60, 0.65)	-0.21 (-1.64, 1.20)
Ever smokers, men / women	-20.10 (-51.00, 10.02)	10.06 (-31.78, 52.25)	-18.67 (-53.26, 15.24)	12.87 (-34.21, 58.71)	-0.24 (-1.06, 0.60)	0.04 (-1.07, 1.17)
Fungicides	-13.68 (-41.81, 14.97)		-11.45 (-43.33, 20.68)		-0.17 (-0.97, 0.61)	
Never smokers, men / women	-22.51 (-72.85, 28.73)	28.10 (-41.07, 98.08)	-12.06 (-67.76, 43.25)	38.65 (-39.70, 114.70)	-0.46 (-1.98, 1.03)	0.03 (-1.88, 1.96)
Ever smokers, men / women	-28.92 (-66.48, 8.72)	21.69 (-36.93, 79.10)	-29.25 (-69.81, 12.87)	21.37 (-45.18, 87.03)	-0.26 (-1.31, 0.77)	0.23 (-1.37, 1.85)
All pesticides	-10.74 (-32.37, 10.90)		-6.60 (-31.35, 18.95)		-0.20 (-0.78, 0.39)	

	FEV1 (ml)		FVC (ml)		FEV1FVC (%)	
Never smokers, men / women	-16.23 (-55.54, 22.68)	17.02 (-34.02, 66.51)	-7.92 (-52.14, 36.53)	27.85 (-26.88, 84.30)	-0.42 (-1.50, 0.69)	-0.18 (-1.58, 1.25)
Ever smokers, men / women	-22.87 (-52.79, 7.68)	10.42 (-30.63, 51.27)	-21.70 (-55.53, 11.46)	14.28 (-32.80, 59.60)	-0.23 (-1.00, 0.58)	0.01 (-1.05, 1.12)
Aromatic solvents	0.28 (-22.53, 23.42)		3.90 (-21.95, 29.65)		0.01 (-0.61, 0.63)	
Never smokers, men / women	-12.82 (-49.46, 24.15)	40.35 (-32.52, 113.02)	-13.70 (-53.99, 25.40)	79.39 (-1.55, 159.07)	-0.08 (-1.18, 1.02)	-0.17 (-2.14, 1.82)
Ever smokers, men / women	-2.34 (-30.95, 26.43)	51.06 (-15.10, 117.27)	-3.31 (-36.96, 28.35)	**89.92 (14.99, 162.53)**	0.07 (-0.71, 0.84)	-0.02 (-1.77, 1.81)
Chlorinated solvents	-16.98 (-34.20, 0.43)		-14.59 (-33.52, 4.42)		-0.18 (-0.64, 0.29)	
Never smokers, men / women	-13.31 (-42.06, 14.73)	5.43 (-61.00, 72.39)	-10.47 (-40.24, 21.30)	31.80 (-42.25, 106.20)	-0.19 (-1.01, 0.64)	-0.14 (-2.00, 1.79)
Ever smokers, men / women	-20.45 (-40.67, 0.17)	-1.38 (-67.59, 65.47)	-20.98 (-43.51, 1.47)	21.02 (-51.04, 93.25)	-0.19 (-0.73, 0.37)	-0.14 (-1.89, 1.67)
Other solvents	-6.05 (-23.61, 11.37)		7.65 (-11.39, 27.28)		-0.24 (-0.70, 0.24)	
Never smokers, men / women	3.67 (-28.12, 36.60)	-11.05 (-46.12, 24.35)	22.33 (-14.03, 58.55)	17.37 (-22.07, 55.83)	-0.32 (-1.25, 0.61)	-0.56 (-1.53, 0.39)
Ever smokers, men / women	-0.76 (-26.90, 26.20)	-15.35 (-43.67, 12.26)	5.32 (-23.18, 36.10)	-0.01 (-32.23, 32.14)	-0.06 (-0.73, 0.63)	-0.29 (-1.04, 0.44)
Metals	**-18.73 (-34.41, -2.60)**		-10.17 (-27.46, 7.85)		-0.36 (-0.79, 0.07)	
Never smokers, men / women	-11.75 (-37.20, 14.07)	-30.34 (-110.04, 48.64)	0.82 (-27.55, 29.57)	-36.03 (-123.98, 50.88)	-0.47 (-1.20, 0.26)	-0.43 (-2.59, 1.81)
Ever smokers, men / women	**-21.37 (-40.42, -2.68)**	-39.73 (-118.34, 35.47)	-13.01 (-34.08, 8.36)	-49.46 (-135.45, 35.93)	-0.30 (-0.84, 0.21)	-0.24 (-2.38, 1.91)

*Results are posterior medians and 95% CrI in parentheses. Numbers represent absolute change in ml for FEV1 and FVC, and relative %change for FEV1/FVC. A negative sign indicates a lower value compared to fully unexposed to the respective agent. Adjusted for age, sex, current smoking, cumulative smoking pack-years, socioeconomic status and early life disadvantage score. All lung function measurements are without bronchodilation.

MARKED-UP VERSION FOR REVISION

Cumulative occupational exposures and lung function decline in two large general population cohorts

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Abstract

Rationale: Few longitudinal studies have assessed the relationship between occupational exposures and lung function decline in the general population, with sufficiently long follow-up.

Objectives: Our objective was to examine this potential association in two large cohorts (ECRHS and SAPALDIA).

Methods: General population samples aged 18 to 62 were randomly selected in 1991-1993, and followed up approximately 10 and 20 years later. Spirometry (without bronchodilation) was performed at each visit. Coded complete job histories during follow-up visits were linked to a Job-Exposure Matrix, generating cumulative exposure estimates for 12 occupational exposures. FEV_1 and FVC were jointly modelled in linear mixed-effects models, fitted in a Bayesian framework, taking into account age and smoking.

Main results: A total of 40,024 lung function measurements from 17,833 study participants were analyzed. We found accelerated declines in FEV_1 and the FEV_1/FVC ratio for exposure to biological dust, mineral dust and metals (FEV_1 -15.1ml, -14.4ml and -18.7ml respectively, and FEV_1/FVC -0.52%, -0.43% and -0.36% respectively, per 25 intensity-years of exposure). These declines were comparable in magnitude to those associated with long-term smoking. No effect modification by sex or smoking status was identified. Findings were similar between the ECRHS and SAPALDIA cohorts.

Conclusions: Our results greatly strengthen the evidence base implicating occupation, independent of smoking, as a risk factor for lung function decline and by inference for COPD., ~~which is independent from smoking, and~~ This highlights the need to prevent or control these exposures in the workplace.

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Introduction

Chronic Obstructive Pulmonary Disease (COPD) is an important cause of population morbidity and mortality, characterized by a low level of lung function and persistent airflow limitation (1). The most ~~important~~well-recognized risk factor is tobacco smoking, which is associated with the ~~vast~~ majority of COPD cases. Other environmental risk factors are also implicated in the pathogenesis of COPD (2), including occupational exposures (3). The European Community Respiratory Health Survey (ECRHS) has recently shown a higher incidence of COPD in workers exposed to biological dusts, gases, fumes and pesticides, with a combined population attributable fraction of 21% (4).

Lung function declines naturally with age, but an accelerated decline is a primary, though not obligate, cause of COPD (5), as well as a long-term feature of asthma (6). Despite a large number of studies, both population- and industry based, demonstrating an association of asthma and COPD with occupational exposures (3, 7, 8), relatively few population-based longitudinal studies have examined the relationship between lung function decline and occupational exposures as estimated by a Job-Exposure Matrix (JEM) (9–11). A previous analysis from the first 10-year follow-up of the European Community Respiratory Health Survey (ECRHS) did not show a steeper decline in lung function in people exposed to vapors, gases, dusts or fumes (9); the cohort however was fairly young then (30-55 years old at the time), and the follow-up time may have been too short to detect an association.

Therefore our aim was to examine the association between occupational exposures and the rate of lung function decline, given its relevance to COPD risk. To do so, we combined ~~in~~ two large prospective cohorts participating in the ALEC project (www.alecstudy.org): the ECRHS (12) and the Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults (SAPALDIA) (13). Both cohorts have accumulated two decades of follow-up, with more

participants over 50 years of age, thus allowing better and more precise estimates of the determinants of lung function decline. In addition, we examined whether the association between occupation and lung function decline is different between men and women, and between smokers and non-smokers.

Methods

The ECRHS is a multicentre longitudinal study initiated in 1991–1993 that enrolled random general population samples aged 20 to 44 years in 55 centres from 23 countries (12). Participants at baseline (ECRHS I) completed a detailed questionnaire via face-to-face interview and underwent a clinical examination, spirometry and other measurements. They were followed again between 1998 and 2002 (ECRHS II), and a second time between 2010 and 2012 (ECRHS III). SAPALDIA is also a multicentre longitudinal study with objectives, methods and protocols very similar to those of the ECRHS, that was initiated in 1991 (SAPALDIA 1) and enrolled a random general population sample aged 18 to 62 years from eight regions in Switzerland (13). SAPALDIA participants were also followed up in 2001 and 2011 (SAPALDIA 2 and 3). A flowchart of study participants from both cohorts can be found in the online supplement (Supplemental Figure 1).

During both follow-ups, participants in both cohorts were asked to provide a detailed list of their occupations and industries from all jobs held since the last study visit; these were recorded in free text and subsequently coded in the International Classification of Occupations-88 (ISCO-88) standard by trained local coders, who were blind to participant status. Ethical approval for each centre was obtained from their respective competent bodies, and written informed consent was obtained from all participants.

The population for this analysis includes all participants who completed spirometry at baseline (ECRHS I / SAPALDIA 1) and were followed up at least once (at ECRHS II / SAPALDIA 2 and/or ECRHS III / SAPALDIA 3). All spirometries were performed without bronchodilation and according to the ATS/ERS standards for reproducibility, utilizing the maximum Forced Volume Capacity (FVC) and Forced Expiratory Volume in 1 second (FEV_1) per participant. Occupational exposures were determined by linking the participants ISCO-88 coded occupations to the semi-quantitative ALOHA(+) JEM (10). This JEM assigns, for every ISCO-88 job code, three grades of exposure (none, low, high) to twelve agents (biological dusts, mineral dusts, gases/fumes, herbicides, insecticides, fungicides, aromatic solvents, chlorinated solvents, other solvents, and metals) including two composites of the above (All pesticides, and Vapors/Gases/Dusts/Fumes, VGDF). For each participant a cumulative exposure to each agent in intensity-years was calculated, by multiplying the duration of each job with the intensity of exposure (0 for none, 1 for low, and 4 for high); these intensities reflect the lognormal distribution of occupational exposure concentrations when calculating cumulative exposures.

Covariates used for adjustment included at each visit age, sex, height (including its square, to allow for non-linear associations), current asthma, current smoking, lifetime smoking pack-years, Socioeconomic Status (SES) and early life disadvantage score; the latter is a composite variable that includes maternal smoking, maternal asthma, paternal asthma, childhood asthma (before age 10), and having a serious respiratory infection before age 5 (14). Current asthma was defined as a positive response to either of the following three questions: “have you had an attack of asthma in the last 12 months?”, “are you currently taking any medicines for asthma?” and “have you been woken by an attack of shortness of breath at any time in the last 12 months?”. SES was defined according to the participants' age of completion of formal

education, and classified into three categories: high (>19 years), middle (16-19 years), low (<16 years).

Associations between cumulative occupational exposures and lung function (FEV_1, FVC and the FEV_1/FVC ratio) were assessed using linear mixed-effects models, providing the mean change in lung function per intensity-year of exposure. FEV_1 and FVC were jointly modelled, and all models included participant-level random intercepts and slopes, taking account of the correlations both between random intercepts and slopes as well as between FEV_1 and FVC. For each ALOHA(+) exposure agent we fitted two joint models, one using absolute FEV_1 and FVC ("linear model") and one using their logarithms as the outcome ("log-linear model"); from the latter we calculated the effects of exposures on the FEV_1/FVC ratio, as the difference between model parameters for log(FEV_1) and log(FVC). In all instances, the comparison was between exposed and unexposed participants to the particular ALOHA(+) agent, not against participants without any occupational exposure.

The models were fitted in a Bayesian framework with the JAGS software, setting non-informative priors for all parameters, and using 4 chains and 20,000 iterations per chain, discarding the first 2,000 as burn-in, and with a thinning interval of 5. Convergence was checked by visual inspection of the MCMC traceplots and by the Gelman-Rubin statistic. Furthermore, all models included a fully Bayesian imputation sub-model for handling item (covariate) missingness, with hyperparameters set to non-informative priors (see online supplement for details). Uncertainty for the fixed effects was expressed with 95% Credible Intervals (95% CrI).

In addition to the unstratified models, we fitted another set of models with added interaction terms for sex, and smoking status (ever/never smoker), thereby calculating stratified estimates for the effect of occupational exposures in men and women, and in ever smokers and never

smokers. As a criterion to determine the presence of interaction (effect modification) we used the posterior probability distribution of the interaction term, at least 95% of which should lie above or below zero. We also fitted all models separately to the data from each cohort (ECRHS and SAPALDIA), as a sensitivity analysis.

All analyses were performed with the R statistical environment, version 3.6.0 (15).

Results

Table 1 highlights the characteristics of the study population. We analyzed a total of 40,024 lung function measurements from 17,833 study participants across 38 ECRHS and SAPALDIA centres who completed at least one follow-up visit after baseline, with a mean follow-up duration of 16.3 years (range 4.3–22.6 years). Of these participants, 10,803 (60.6%) completed both visits over a mean duration of 19.6 years (range 15.7–22.6 years). 5,793 (32.5%) participants completed the initial and first follow-up visits only, while 1,237 (6.9%) completed the initial and second follow-up visits only. Slightly less than half of our sample had never smoked, and about a third were current smokers at baseline, dropping almost by half to 18.3% at the second follow-up. A little less than half of all participants had been occupationally exposed to VGDF at some point during follow-up (39.8%), whereas fewer had been exposed to solvents (24.2%), metals (9.3%) or pesticides (3.3%). Men were overall more likely than women to be occupationally exposed to most agents, with the exception of biological dust (Table 2). In addition, many exposures showed substantial overlap with each other (Figure 1). A list of the most common jobs by exposure category can be found online (Supplemental Table 1).

Lifetime smoking pack-years were missing in 7.8% of all observations, and had to be imputed in our models as described in the "Methods" section; also current smoking status was missing in 2.9%, current asthma in 0.5% and SES in 1.1% of all observations. Lung function in the study population naturally declined with advancing age across both follow-ups, and our Bayesian mixed-effects model was reliable in describing both mean lung function by age as well as the variability around the mean (Figure 2).

Table 3 summarizes the main results from our analysis, i.e. the effect of 25 intensity-years of occupational exposure to each ALOHA(+) agent on the three lung function parameters (FEV_1, FVC and the FEV_1/FVC ratio), both overall and stratified by sex and smoking status. A negative sign indicates reduced lung function compare to no occupational exposure to the respective agent given the same age, sex and other covariates, i.e. an accelerated lung function decline, whereas a positive number indicates a slower lung function decline. As the numbers are very small, the effect per 25 intensity-years is presented rather than per one intensity-year of exposure.

A decreased FEV_1/FVC ratio was observed for biological dust and mineral dust exposure (-0.52% and -0.43% respectively, Table 3), which was purely attributed to a FEV_1 decline (-15.08 ml and -14.42 ml respectively) with no change in FVC. A significant decline in FEV_1 only was also observed for metals exposure (-18.73 ml, 95% CrI -34.41 to -2.60 ml), as well as a lower FEV_1/FVC ratio for the composite VGDF exposure (-0.34%, 95% CrI -0.56 to -0.12%). On the other hand, smaller lung function declines were seen for gases & fumes (-7.35 ml in FEV_1 and -0.24% in FEV_1/FVC), and also for pesticides, especially in terms of FEV_1, although the results were very imprecise, with a wider 95% CrI than other exposures (Table 3). Among solvents, only exposure to chlorinated solvents was weakly associated with both lower FEV_1 and lower FVC (-16.98 ml and -14.59 ml). For comparison, as estimated in the

model, 25 pack-years of smoking reduced FEV1 by an additional -11.07 ml (95% CrI -22.27 to 2.49 ml), FVC by -14.83 ml (95% CrI -32.29 to -0.55 ml) and FEV1/FVC by -0.21% (95% CrI -0.44 to 0.03%). Also, current asthma was associated with a FEV1 that was -80.28 ml lower (95% CrI -92.72 to -67.71 ml), a FVC lower by -13.60 ml (95% CrI -27.44 to -0.05 ml) and a FEV1/FVC -2.16% lower (95% CrI -2.52 to -1.81%).

No effect modification by sex was detected for any occupational exposure and any lung function parameter, with one exception: women exposed to aromatic solvents had a slower FVC decline than unexposed women (+86.87 ml, 95% CrI 13.80 to 161.25 ml), whereas no significant difference was found among men (-7.84 ml, 95% CrI -34.96 to 20.18 ml). In addition, no effect modification by smoking status was detected, although in most cases the effects of occupational exposures in ever smokers tended to be slightly lower than in never smokers (Table 3). In the sensitivity analysis, results were similar between the two cohorts and showed wide overlap; pesticide exposure resulted in greater FEV_1 decline among ECRHS participants, while solvents resulted in slightly greater declines among SAPALDIA participants (Supplemental~~ry~~ Table ~~1~~2).

Discussion

In this pooled analysis from two large longitudinal cohorts we showed that certain groups of occupational exposures, namely biological dust, mineral dust and metals exposure, were prospectively associated with accelerated lung function decline, specifically with respect to FEV1 and the FEV1/FVC ratio. This loss of lung function is comparable to that associated with smoking, highlighting the importance of these occupational exposures in respiratory health. Our study provides significant new evidence on the topic, as few such studies have

examined longitudinal lung function decline and in a general population setting (10, 11, 16–18). In comparison to industry-based studies, general population cohorts can provide more generalizable information by including all types of exposures across all industries, and by adjusting for socioeconomic status and other covariates. Previous analyses from both ECRHS and SAPALDIA have shown an increased asthma risk for certain occupational exposures (19), an increased incidence of COPD in participants occupationally exposed to biological dust and mineral dust (4, 20), as well as an increased incidence of chronic cough and chronic phlegm symptoms in those exposed to mineral dust and metals (21). Our current analysis corroborates previous findings for biological dust in a much larger study population with additional follow-up. It also indicates that mineral dust and metals exposure are associated also with lung function decline and not only with chronic bronchitis symptoms. The latter result is in agreement with recent findings from the smaller Tasmanian longitudinal Health Study (TAHS) cohort (22), as well as with a systematic review of exposure to welding fumes and lung function decline (23).

A striking finding is the very small absolute effect sizes observed, only a few millilitres (or a fraction of a percentage point) of lung function decline after 25 intensity-years of exposure, which for many workers may represent their entire lifetime exposure. At first glance such reductions could not plausibly account for an increased COPD risk as seen in previous analyses (4, 8, 20); however, these are average subject-specific effects over an exposure group, and small average reductions can mask a larger effect for certain individuals inside the group (24), especially if one also considers the sizeable individual variation in lung function (Figure 2). In addition, the comparator is not a fully occupationally unexposed group, but rather participants unexposed to the agent under consideration (who may have other occupational exposures). Furthermore, non-differential misclassification is the main limitation

in using JEMs in population-based occupational epidemiology studies, mostly stemming from the variability in job tasks and actual exposures between workers belonging in the same JEM exposure category (25). Grouping exposures in a JEM does not in itself bias regression coefficients towards the null, but rather results in imprecision due to Berkson-type error (26); nevertheless, regression dilution bias can be introduced if the estimated group mean is different than the true group mean (27). Intra-individual variability in exposure over time is another consideration, which is particularly relevant when calculating a cumulative exposure estimate based on job duration and a JEM classification, in this case over a long period of 20 years (28). All these factors mean that the small average declines observed in our analysis should not be used for prediction, as they are unlikely to represent the actual magnitude of the effect of these exposures on lung function, in any particular individual. For the same reasons, our results cannot definitively rule out an effect of occupational solvent exposure on lung function as recently observed in the TAHS cohort (22), nor an effect of pesticide exposure (4, 10). Especially as regards pesticides, the small percentage of exposed participants (less than 5% – Table 2) has substantially reduced precision, as reflected in the wider credible intervals of the lung function decline estimates.

Smoking is known to induce inflammation and impair the host defense mechanisms of the lung (29, 30), and has been reported to increase the adverse effect of occupational exposures on lung function decline and COPD risk (31–33). However, in our analyses we found little evidence of effect modification by smoking, after tightly adjusting both for current smoking status and cumulative pack-years. This indicates that all workers may be at risk of airway obstruction due to these occupational exposures, even though this may be more clinically significant for smokers, who represent the majority of COPD patients in most countries, and who already have lower levels of lung function and any additional loss or acceleration of

decline may push them over the diagnostic cutoff. Similarly, we found little difference between men and women on the effect of occupational exposures, even though the distribution of jobs per each exposure category was different between men and women. This may partially be explained by the lower proportion of occupationally exposed women in our cohorts, particularly for exposures like metals, pesticides and solvents. Future occupational epidemiology studies should try to recruit more women, in order to investigate effect modification by sex.

Strengths of the current study include its prospective population-based design and long follow-up of 20 years. Full job histories were collected for the study period and cumulative occupational exposures were calculated using a JEM instead of self-report; the latter could be vulnerable to recall bias, especially given the long follow-up involved. Lung function was modelled in great detail, using a mixed-effects model with random intercepts and slopes, and accounting for the correlation between FEV1 and FVC; joint modelling of both spirometric parameters is important, as we identified positive correlations not just between the participant-level random intercepts and slopes for FEV1 and for FVC, but also between the intercepts for FEV1 and FVC and the slopes for FEV1 and FVC (data not shown). In addition we controlled for multiple confounders, including socioeconomic status, current asthma and especially lifetime smoking pack-years, in order to minimize confounding by intensity of smoking. The sample size was very large, thanks to the pooling of two large cohorts, facilitating the detection of associations of very small magnitude.

On the other hand, there are certain limitations to our study. Spirometries were performed without bronchodilation, and the results should be interpreted accordingly. Accelerated lung function decline in and of itself does not equate with COPD risk; in particular, ~~As~~ occupation ~~is~~can also be a risk factor for asthma, thus it is not possible to distinguish lung function

decline due to new-onset occupational asthma from that contributing to increased COPD incidence. The application of a JEM may ensure more objective exposure estimates, free from exposure-specific recall, but can introduce both imprecision due to Berkson-type error and some bias towards the null; therefore, despite the large sample size, it may not have been always sufficient to detect an association, e.g. for the effects of pesticides or to identify effect modification. For the same reason, we cannot disentangle the effect of multiple overlapping exposures, which would require a large number of small subgroup analyses. We could also not assess heterogeneity of the results across study centres or countries, since the (necessary) inclusion of participant-level random effects left almost no variance to be explained by study centre. Finally, residual confounding cannot be completely ruled out despite the detailed model adjustments, and may have affected the observed associations given their small magnitude. As this is an observational study, some selection and response bias also cannot be definitely ruled out.

In conclusion, long-term occupational exposure to biological dust, mineral dust and metals over two decades of follow-up was associated with an accelerated decline in FEV_1 and the FEV_1/FVC ratio, which would translate to an increased risk of airways obstruction and COPD. This decline was comparable to that associated with smoking, and similar between men and women as well as between smokers and non-smokers. These results strengthen the case for occupation as a modifiable risk factor for asthma and COPD, in agreement with previous studies. And they make the case for avoiding these exposures in the workplace, or controlling them with appropriate protective measures, in order to protect the respiratory health of workers.

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Figure Legends

Figure 1: Correlation matrix (Spearman's rho) between cumulative occupational exposures (intensity-years) in the study population, as estimated by the ALOHA(+) JEM. (N=17,833 ECRHS and SAPALDIA participants)

Figure 2: Lung function by age and sex in the study population, observed and model-predicted. (N=40,024 measurements in 17,833 ECRHS and SAPALDIA participants)

Footnote for Figure 2:

Height set to cohort mean by sex; smoking pack-years and cumulative VGDF exposure set to cohort mean by age and sex.

Table 1: Characteristics of study participants, by study wave. (N=17,833 ECRHS and SAPALDIA participants followed-up at least once)*

	ECRHS I / SAPALDIA 1	ECRHS II / SAPALDIA 2	ECRHS III / SAPALDIA 3
Number of participants	17833 (9765 / 8068)	16596 (8725 / 7871)	12040 (6013 / 6027)
% men	48.0 (48.0 / 47.9)	48.1 (48.1 / 48.0)	47.7 (47.9 / 47.5)
Mean age	37.2 (34.0 / 41.0)	47.2 (42.8 / 52.0)	56.9 (54.1 / 59.7)
% current asthma	6.4 (7.9 / 4.7)	7.9 (9.9 / 5.6)	7.6 (11.2 / 4.1)
% never smokers	46.0 (44.8 / 47.4)	45.9 (45.5 / 46.4)	48.5 (49.0 / 48.1)
% current smokers	33.3 (34.5 / 31.9)	27.7 (29.0 / 26.3)	18.3 (18.1 / 18.5)
Mean cumulative smoking pack-years	8.4 (7.2 / 10.0)	11.1 (9.7 / 12.7)	11.7 (10.9 / 12.3)
% of participants exposed			
Biological dust	-	22.0 (26.6 / 17.0)	26.7 (30.7 / 22.6)
Mineral Dust	-	17.6 (21.1 / 13.8)	20.9 (23.9 / 18.0)
Gases & fumes	-	31.7 (37.4 / 25.4)	37.4 (41.3 / 33.5)
Vapors, Gases, Dusts & Fumes	-	35.8 (41.9 / 29.2)	42.0 (46.1 / 37.9)
Herbicides	-	1.7 (1.5 / 1.9)	2.2 (1.8 / 2.6)
Insecticides	-	2.1 (2.3 / 1.9)	2.8 (2.9 / 2.6)
Fungicides	-	2.2 (2.4 / 2.0)	3.1 (3.4 / 2.8)
All pesticides	-	2.7 (3.2 / 2.1)	3.6 (4.3 / 3.0)
Aromatic solvents	-	11.4 (13.1 / 9.6)	13.5 (14.9 / 12.1)
Chlorinated solvents	-	9.1 (10.3 / 7.7)	10.9 (12.2 / 9.6)
Other solvents	-	19.4 (23.0 / 15.3)	23.6 (26.9 / 20.4)
Metals	-	7.9 (9.5 / 6.1)	9.7 (11.4 / 8.0)
Mean cumulative exposures since previous follow-up (intensity-years)			
Biological dust	-	2.2 (2.2 / 2.1)	4.1 (4.7 / 3.6)
Mineral Dust	-	2.3 (2.4 / 2.2)	4.1 (4.7 / 3.5)
Gases & fumes	-	3.6 (3.9 / 3.3)	6.6 (7.7 / 5.4)
Vapors, Gases, Dusts & Fumes	-	5.1 (5.3 / 4.8)	9.2 (10.6 / 7.9)
Herbicides	-	0.2 (0.1 / 0.3)	0.4 (0.3 / 0.6)
Insecticides	-	0.4 (0.3 / 0.6)	0.8 (0.6 / 1.1)
Fungicides	-	0.3 (0.3 / 0.4)	0.6 (0.6 / 0.6)
All pesticides	-	0.5 (0.4 / 0.6)	0.9 (0.7 / 1.1)
Aromatic solvents	-	1.0 (1.0 / 1.0)	1.7 (1.9 / 1.6)
Chlorinated solvents	-	1.2 (1.2 / 1.2)	1.9 (2.1 / 1.7)
Other solvents	-	1.7 (1.8 / 1.6)	3.2 (3.7 / 2.7)
Metals	-	1.2 (1.3 / 1.1)	2.0 (2.3 / 1.7)

* Data for the entire cohort (data for ECRHS / data for SAPALDIA)

Table 2: Proportion of participants with any occupational exposure during follow-up, stratified by sex. (N=17,833 ECRHS and SAPALDIA participants followed-up at least once)*

	% of men exposed	% of women exposed
Biological dust	22.2 (25.5 / 18.2)	27.7 (32.5 / 22.0)
Mineral Dust	28.8 (33.6 / 23.1)	11.8 (13.4 / 10.0)
Gases & fumes	42.7 (47.9 / 36.4)	29.0 (33.2 / 23.8)
Vapors, Gases, Dusts & Fumes	46.3 (51.8 / 39.7)	33.8 (38.2 / 28.4)
Herbicides	2.8 (2.5 / 3.1)	1.2 (1.0 / 1.5)
Insecticides	3.5 (3.8 / 3.1)	1.6 (1.6 / 1.5)
Fungicides	4.1 (4.7 / 3.4)	1.5 (1.4 / 1.6)
All pesticides	4.9 (5.9 / 3.7)	1.7 (1.8 / 1.6)
Aromatic solvents	21.5 (23.6 / 18.8)	5.0 (5.9 / 3.8)
Chlorinated solvents	16.8 (18.7 / 14.5)	4.5 (5.2 / 3.7)
Other solvents	25.5 (28.4 / 22.0)	18.9 (22.7 / 14.4)
Metals	17.3 (20.1 / 13.9)	1.8 (2.4 / 1.0)

* Data for the entire cohort (data for ECRHS / data for SAPALDIA)

Table 3: Effect of occupational exposures on lung function decline, per 25 intensity-years of exposure, compared to unexposed to the respective agent, overall and stratified by sex and smoking status. (N=17,833 ECRHS and SAPALDIA participants followed-up at least once)*

	FEV1 (ml)		FVC (ml)		FEV1FVC (%)	
Biological dust	**-15.08 (-28.66, -0.88)**		2.68 (-12.68, 18.28)		**-0.52 (-0.88, -0.16)**	
Never smokers, men / women	-3.23 (-26.67, 20.22)	-14.38 (-45.32, 15.59)	13.44 (-12.79, 39.41)	8.35 (-25.83, 43.27)	-0.28 (-0.95, 0.41)	-0.52 (-1.35, 0.36)
Ever smokers, men / women	-15.86 (-33.93, 2.09)	**-27.11 (-53.91, -1.19)**	-0.47 (-20.80, 19.98)	-5.59 (-35.49, 24.14)	**-0.52 (-1.02, -0.02)**	**-0.76 (-1.46, -0.04)**
Mineral Dust	**-14.42 (-26.41, -2.50)**		0.12 (-12.99, 13.10)		**-0.43 (-0.75, -0.10)**	
Never smokers, men / women	-9.38 (-28.04, 9.65)	5.55 (-29.53, 39.95)	10.96 (-10.24, 31.96)	15.54 (-22.24, 53.37)	**-0.56 (-1.10, -0.02)**	-0.31 (-1.23, 0.61)
Ever smokers, men / women	**-20.83 (-35.33, -6.25)**	-6.15 (-37.68, 24.57)	-7.12 (-23.55, 9.48)	-2.42 (-37.19, 33.52)	**-0.44 (-0.84, -0.03)**	-0.18 (-1.01, 0.65)
Gases & fumes	-7.35 (-17.99, 3.15)		3.46 (-8.66, 15.25)		-0.24 (-0.53, 0.04)	
Never smokers, men / women	4.22 (-12.83, 21.44)	-10.01 (-39.00, 19.03)	14.43 (-4.90, 33.41)	11.31 (-22.02, 43.81)	-0.14 (-0.64, 0.35)	-0.38 (-1.16, 0.43)
Ever smokers, men / women	-10.22 (-23.34, 3.14)	-24.37 (-51.63, 3.81)	-2.14 (-16.77, 12.32)	-5.52 (-36.24, 25.02)	-0.24 (-0.59, 0.11)	-0.48 (-1.24, 0.24)
Vapors, Gases, Dusts & Fumes	-7.42 (-15.93, 1.03)		6.02 (-3.04, 15.42)		**-0.34 (-0.56, -0.12)**	
Never smokers, men / women	-1.44 (-14.83, 11.91)	-6.21 (-28.65, 16.31)	11.89 (-2.73, 26.67)	14.92 (-10.55, 39.96)	-0.27 (-0.65, 0.11)	-0.47 (-1.06, 0.13)
Ever smokers, men / women	-9.45 (-19.62, 0.97)	-14.20 (-35.47, 7.03)	1.76 (-9.68, 13.41)	5.14 (-18.42, 28.40)	**-0.33 (-0.61, -0.06)**	-0.52 (-1.06, 0.01)
Herbicides	-14.69 (-49.54, 20.57)		-8.57 (-47.42, 29.56)		-0.34 (-1.28, 0.59)	
Never smokers, men / women	-19.16 (-85.70, 43.26)	35.35 (-51.37, 122.19)	-23.73 (-96.58, 48.69)	22.63 (-73.97, 120.70)	-0.28 (-2.17, 1.65)	0.55 (-1.85, 3.00)
Ever smokers, men / women	-33.13 (-77.76, 11.56)	21.78 (-46.93, 91.26)	-20.84 (-72.01, 28.90)	26.82 (-50.71, 104.30)	-0.67 (-1.88, 0.55)	0.12 (-1.71, 2.05)
Insecticides	-7.24 (-29.73, 15.04)		-2.43 (-27.68, 23.16)		-0.23 (-0.83, 0.38)	
Never smokers, men / women	-7.72 (-47.54, 32.36)	22.94 (-28.06, 74.16)	3.48 (-40.41, 46.91)	35.24 (-22.53, 92.33)	-0.50 (-1.60, 0.65)	-0.21 (-1.64, 1.20)
Ever smokers, men / women	-20.10 (-51.00, 10.02)	10.06 (-31.78, 52.25)	-18.67 (-53.26, 15.24)	12.87 (-34.21, 58.71)	-0.24 (-1.06, 0.60)	0.04 (-1.07, 1.17)
Fungicides	-13.68 (-41.81, 14.97)		-11.45 (-43.33, 20.68)		-0.17 (-0.97, 0.61)	
Never smokers, men / women	-22.51 (-72.85, 28.73)	28.10 (-41.07, 98.08)	-12.06 (-67.76, 43.25)	38.65 (-39.70, 114.70)	-0.46 (-1.98, 1.03)	0.03 (-1.88, 1.96)
Ever smokers, men / women	-28.92 (-66.48, 8.72)	21.69 (-36.93, 79.10)	-29.25 (-69.81, 12.87)	21.37 (-45.18, 87.03)	-0.26 (-1.31, 0.77)	0.23 (-1.37, 1.85)
All pesticides	-10.74 (-32.37, 10.90)		-6.60 (-31.35, 18.95)		-0.20 (-0.78, 0.39)	

	FEV1 (ml)		FVC (ml)		FEV1FVC (%)	
Never smokers, men / women	-16.23 (-55.54, 22.68)	17.02 (-34.02, 66.51)	-7.92 (-52.14, 36.53)	27.85 (-26.88, 84.30)	-0.42 (-1.50, 0.69)	-0.18 (-1.58, 1.25)
Ever smokers, men / women	-22.87 (-52.79, 7.68)	10.42 (-30.63, 51.27)	-21.70 (-55.53, 11.46)	14.28 (-32.80, 59.60)	-0.23 (-1.00, 0.58)	0.01 (-1.05, 1.12)
Aromatic solvents	0.28 (-22.53, 23.42)		3.90 (-21.95, 29.65)		0.01 (-0.61, 0.63)	
Never smokers, men / women	-12.82 (-49.46, 24.15)	40.35 (-32.52, 113.02)	-13.70 (-53.99, 25.40)	79.39 (-1.55, 159.07)	-0.08 (-1.18, 1.02)	-0.17 (-2.14, 1.82)
Ever smokers, men / women	-2.34 (-30.95, 26.43)	51.06 (-15.10, 117.27)	-3.31 (-36.96, 28.35)	**89.92 (14.99, 162.53)**	0.07 (-0.71, 0.84)	-0.02 (-1.77, 1.81)
Chlorinated solvents	-16.98 (-34.20, 0.43)		-14.59 (-33.52, 4.42)		-0.18 (-0.64, 0.29)	
Never smokers, men / women	-13.31 (-42.06, 14.73)	5.43 (-61.00, 72.39)	-10.47 (-40.24, 21.30)	31.80 (-42.25, 106.20)	-0.19 (-1.01, 0.64)	-0.14 (-2.00, 1.79)
Ever smokers, men / women	-20.45 (-40.67, 0.17)	-1.38 (-67.59, 65.47)	-20.98 (-43.51, 1.47)	21.02 (-51.04, 93.25)	-0.19 (-0.73, 0.37)	-0.14 (-1.89, 1.67)
Other solvents	-6.05 (-23.61, 11.37)		7.65 (-11.39, 27.28)		-0.24 (-0.70, 0.24)	
Never smokers, men / women	3.67 (-28.12, 36.60)	-11.05 (-46.12, 24.35)	22.33 (-14.03, 58.55)	17.37 (-22.07, 55.83)	-0.32 (-1.25, 0.61)	-0.56 (-1.53, 0.39)
Ever smokers, men / women	-0.76 (-26.90, 26.20)	-15.35 (-43.67, 12.26)	5.32 (-23.18, 36.10)	-0.01 (-32.23, 32.14)	-0.06 (-0.73, 0.63)	-0.29 (-1.04, 0.44)
Metals	**-18.73 (-34.41, -2.60)**		-10.17 (-27.46, 7.85)		-0.36 (-0.79, 0.07)	
Never smokers, men / women	-11.75 (-37.20, 14.07)	-30.34 (-110.04, 48.64)	0.82 (-27.55, 29.57)	-36.03 (-123.98, 50.88)	-0.47 (-1.20, 0.26)	-0.43 (-2.59, 1.81)
Ever smokers, men / women	**-21.37 (-40.42, -2.68)**	-39.73 (-118.34, 35.47)	-13.01 (-34.08, 8.36)	-49.46 (-135.45, 35.93)	-0.30 (-0.84, 0.21)	-0.24 (-2.38, 1.91)

*Results are posterior medians and 95% CrI in parentheses. Numbers represent absolute change in ml for FEV1 and FVC, and relative %change for FEV1/FVC. A negative sign indicates a lower value compared to fully unexposed to the respective agent. Adjusted for age, sex, current smoking, cumulative smoking pack-years, socioeconomic status and early life disadvantage score. All lung function measurements are without bronchodilation.

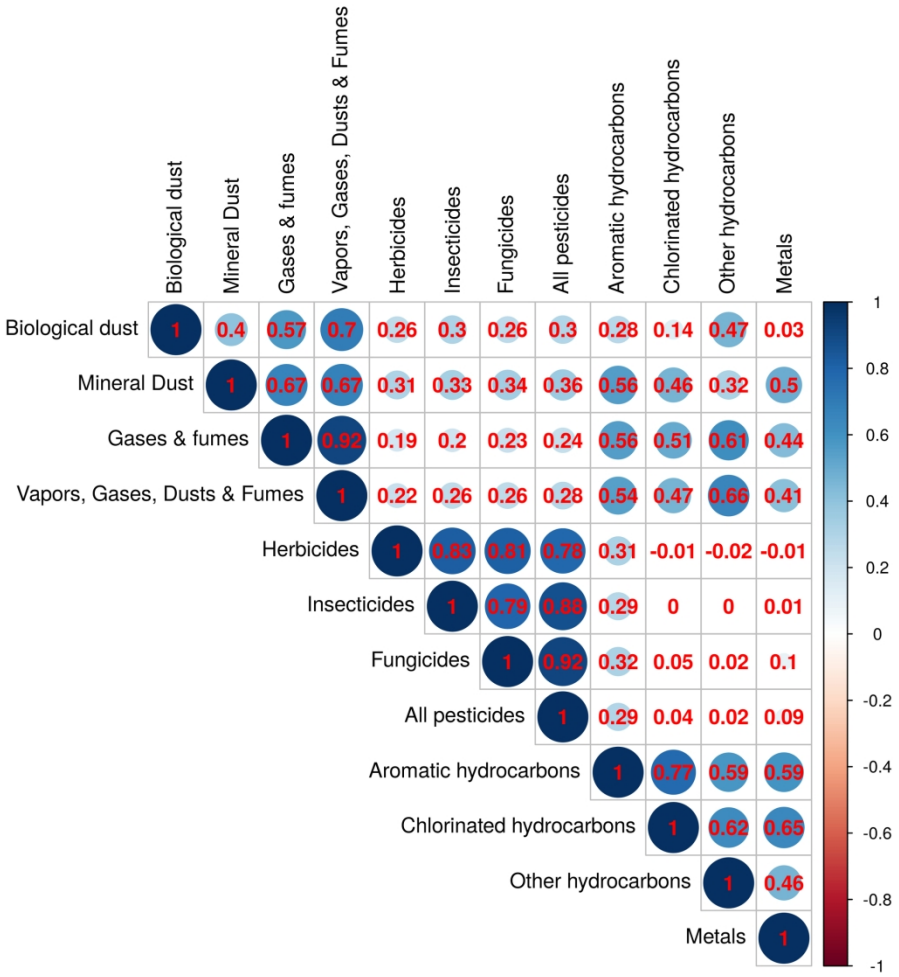


Figure 1: Correlation matrix (Spearman's rho) between cumulative occupational exposures (intensity-years) in the study population, as estimated by the ALOHA(+) JEM. (N=17,833 ECRHS and SAPALDIA participants)

203x203mm (300 x 300 DPI)

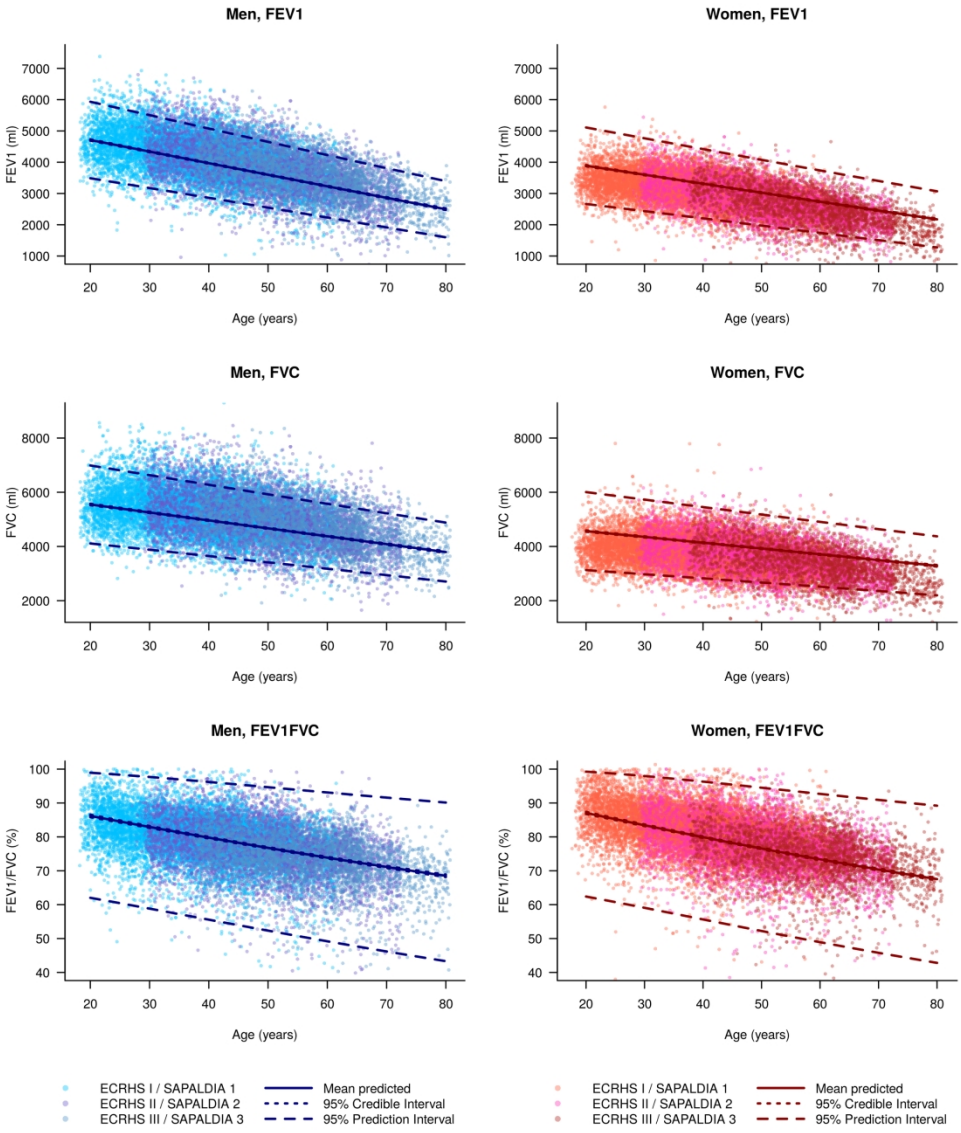


Figure 2: Lung function by age and sex in the study population, observed and model-predicted. (N=40,024 measurements in 17,833 ECRHS and SAPALDIA participants)

Footnote for Figure 2: Height set to cohort mean by sex; smoking pack-years and cumulative VGDF exposure set to cohort mean by age and sex.

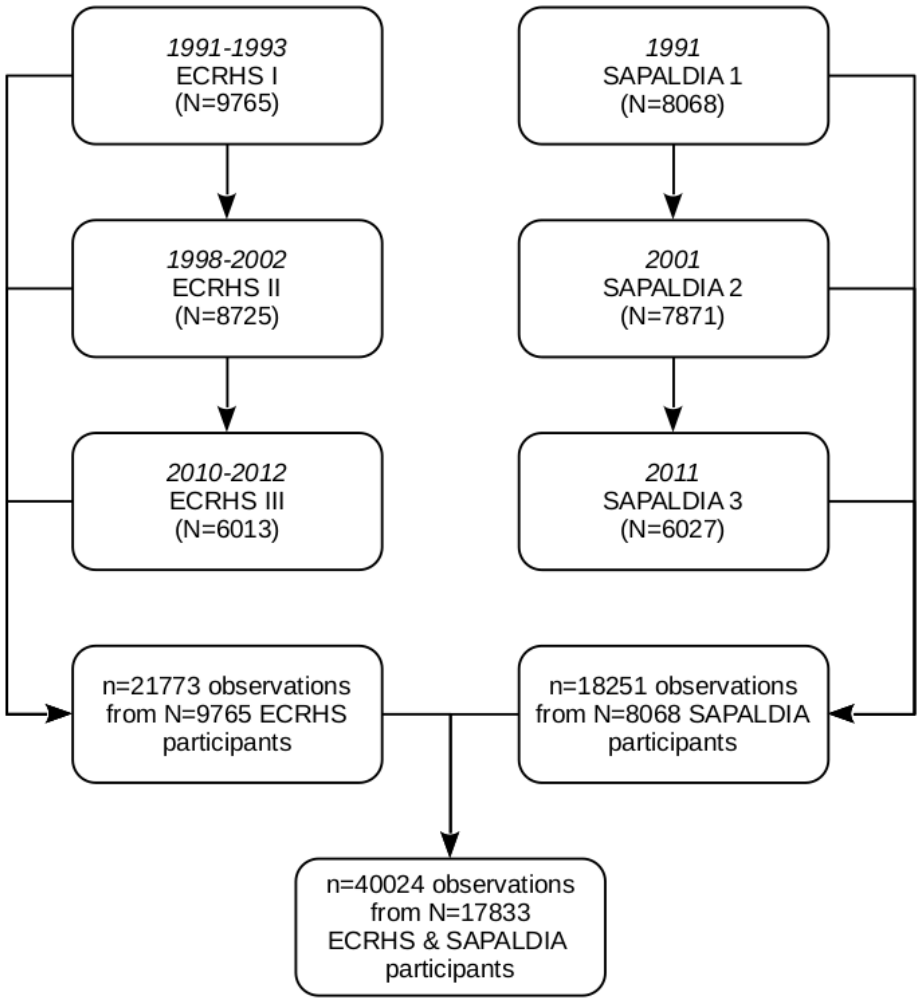
217x254mm (300 x 300 DPI)

ONLINE DATA SUPPLEMENT

Cumulative occupational exposures and lung function decline in two large general population cohorts

Theodore Lytras, Anna Beckmeyer-Borowko, Manolis Kogevinas, Hans Kromhout, Anne-Elie Carsin, Josep Maria Antó, Hayat Bentouhami, Joost Weyler, Joachim Heinrich, Dennis Nowak, Isabel Urrutia, Jesús Martínez-Moratalla, José Antonio Gullón, Antonio Pereira Vega, Chantal Raherison Semjen, Isabelle Pin, Pascal Demoly, Bénédicte Leynaert, Simona Villani, Thorarinn Gislason, Øistein Svanes, Mathias Holm, Bertil Forsberg, Dan Norbäck, Amar J Mehta, Dirk Keidel, David Vernez, Geza Benke, Rain Jõgi, Kjell Torén, Torben Sigsgaard, Vivi Schlünssen, Mario Olivieri, Paul D Blanc, John Watkins, Roberto Bono, Giulia Squillacioti, A. Sonia Buist, Roel Vermeulen, Deborah Jarvis, Nicole Probst-Hensch, Jan-Paul Zock

Supplemental Figure 1: Flowchart of study participants from ECRHS and SAPALDIA



Details about the modelling of lung function

The fixed-effects part of the main model (the one including cumulative occupational exposures) jointly modelled absolute FEV_1 and FVC (or their logarithm) as a function of age and other covariates at each time point. Random intercepts and slopes for every participant were included, both for FEV_1 and FVC; their correlations were modelled via a 4x4 unstructured covariance matrix, using an inverse Wishart prior. Correlation coefficients (Equation 1) were all set on a uniform U(-1,1) prior, all standard deviations (random effects and residual) on uniform priors with a high upper limit, and all fixed-effects coefficients were set on normal priors with zero mean and a high variance.

Equation 1: Correlation matrix between FEV_1 random intercept, FEV_1 random slope, FVC random intercept and FVC random slope.

$$\begin{bmatrix} 1 & \rho_{u1} & \rho_{ua} & \rho_{Q1} \\ \rho_{u1} & 1 & \rho_{Q2} & \rho_{ub} \\ \rho_{ua} & \rho_{Q2} & 1 & \rho_{u2} \\ \rho_{Q1} & \rho_{ub} & \rho_{u2} & 1 \end{bmatrix}$$

Equation 2: Full equation of the model (Y_{ij} : lung function for participant j at time i, $k_{FEV1,}k_{FVC}$: binary indicators for lung function parameter, Z: random effect terms)

$$\begin{aligned} Y_{ij} = {} & k_{FEV1}(\beta_1 + \beta_2 cumexp_{ij} + \beta_3 age_{ij} + \beta_4 female_j age_{ij} + \beta_5 female_j + \beta_6 female_j cumexp_{ij} + \\ & + \beta_7 everSmoked_{ij} cumexp_{ij} + \beta_8 height_j + \beta_9 height_j^2 + \beta_{10} currentSmoker_{ij} + \beta_{11} cumPackYears_{ij} + \\ & + \beta_{12} SES_j^{mid} + \beta_{13} SES_j^{low} + \beta_{14} disadvScore_j + \beta_{15} currentAsthma_{ij}) + \\ & k_{FVC}(\beta_{16} + \beta_{17} cumexp_{ij} + \beta_{18} age_{ij} + \beta_{19} female_j age + \beta_{20} female_j + \beta_{21} female_j cumexp_{ij} + \\ & + \beta_{22} everSmoked_{ij} cumexp_{ij} + \beta_{23} height_j + \beta_{24} height_j^2 + \beta_{25} currentSmoker_{ij} + \beta_{26} cumPackYears_{ij} + \\ & + \beta_{27} SES_j^{mid} + \beta_{28} SES_j^{low} + \beta_{29} disadvScore_j + \beta_{30} currentAsthma_{ij}) + \\ & + k_{FEV1}(Z_{a1} + Z_{b1} age_{ij}) + k_{FVC}(Z_{a2} + Z_{b2} age_{ij}) \end{aligned}$$

As the participant-level random effect already explains a large part of the overall variance, and participants are nested within centres, a centre-level random effect was not needed and not included in the models.

We set priors on three fixed covariates that included some missing values, namely current asthma, SES, current smoking and total smoking pack-years, in order to do Bayesian imputation of their missing values. For current asthma and current smoking we used binomial priors with non-informative Beta(0.5,0.5) hyperpriors. For SES we used a categorical prior with a non-informative Dirichlet(0.5,0.5,0.5) hyperprior. For total smoking pack-years we modelled the smoking pack-years since the previous follow-up as a zero-inflated gamma distribution; we set a binomial prior on the probability of having smoked, and a gamma prior on the number of pack-years conditional on having smoked, with the hyperparameters of the two priors estimated from the observed data at each time point.

The same specification was used for both the linear (absolute FEV_1 and FVC as outcome) and the log-linear ($\log(FEV_1)$ and log(FVC) as outcome) models. From the log-linear model, inference was made on the FEV_1/FVC ratio by taking the difference between model coefficients for $\log(FEV_1)$ and log(FVC); thus, after exponentiation, effect estimates on the FEV_1/FVC ratio are percentages on a relative scale, not absolute differences on a % scale (although the practical difference is very small).

The full JAGS code of the model was as follows (interaction terms for gender and smoking status are included):

```
data {
    zero[1] <- 0
    zero[2] <- 0
    zero[3] <- 0
    zero[4] <- 0

    noninf[1] <- 0.5
    noninf[2] <- 0.5
    noninf[3] <- 0.5
}

model {

  # Random intercept and slope for each participant
  for(j in 1:K ) {
    u1[j,1:4] ~ dmnorm(zero, tau.u)
  }

  # Variance-covariance matrix of the participant random effects
  R.u[1,1] <- pow(sigma.a1, 2)
  R.u[2,2] <- pow(sigma.b1, 2)
  R.u[3,3] <- pow(sigma.a2, 2)
  R.u[4,4] <- pow(sigma.b2, 2)
  R.u[1,2] <- rho.u1 * sigma.a1 * sigma.b1
  R.u[2,1] <- R.u[1,2]
  R.u[3,4] <- rho.u2 * sigma.a2 * sigma.b2
  R.u[4,3] <- R.u[3,4]
  R.u[1,3] <- rho.ua * sigma.a1 * sigma.a2
  R.u[3,1] <- R.u[1,3]
  R.u[2,4] <- rho.ub * sigma.b1 * sigma.b2
  R.u[4,2] <- R.u[2,4]
  R.u[1,4] <- rho.Q1 * sigma.a1 * sigma.b2
  R.u[4,1] <- R.u[1,4]
  R.u[2,3] <- rho.Q2 * sigma.b1 * sigma.a2
  R.u[3,2] <- R.u[2,3]

  tau.u ~ dwish(R.u, 4)
  sigma.u <- inverse(tau.u)
    # with priors:
  sigma.a1 ~ dunif(0, 40)
  sigma.b1 ~ dunif(0, 40)
  sigma.a2 ~ dunif(0, 40)
  sigma.b2 ~ dunif(0, 40)
  rho.u1 ~ dunif(-1,1)
  rho.u2 ~ dunif(-1,1)
  rho.Q2 ~ dunif(-1,1)
  rho.ua <- rho.u1 * rho.Q2
  rho.ub <- rho.Q2 * rho.u2
  rho.Q1 <- rho.u1 * rho.Q2 * rho.u2

  # Define model for each observational unit
  for(i in 1:N ) {
    mu[i] <-    beta[1]*FEV1[i] + beta[2]*FEV1[i]*X[i] + beta[3]*FEV1[i]*age[i] +
                beta[4]*FEV1[i]*age[i]*female[i] +
                beta[5]*FEV1[i]*female[i] + beta[6]*FEV1[i]*X[i]*female[i] +
                beta[7]*FEV1[i]*X[i]*everSmoked[pid[i], survey[i]] +
                beta[8]*FEV1[i]*height[i] + beta[9]*FEV1[i]*height[i]^2 +
                beta[10]*FEV1[i]*cursmoke[i] +
                beta[11]*FEV1[i]*packyrs[pid[i],survey[i]] +
                beta[12]*FEV1[i]*SESmid[pid[i]] + beta[13]*FEV1[i]*SESlow[pid[i]] +
                beta[14]*FEV1[i]*disadv[i] + beta[15]*FEV1[i]*asthma[i] +

                beta[16]*FVC[i] + beta[17]*FVC[i]*X[i] + beta[18]*FVC[i]*age[i] +
                beta[19]*FVC[i]*age[i]*female[i] +
                beta[20]*FVC[i]*female[i] + beta[21]*FVC[i]*X[i]*female[i] +
                beta[22]*FVC[i]*X[i]*everSmoked[pid[i], survey[i]] +
                beta[23]*FVC[i]*height[i] + beta[24]*FVC[i]*height[i]^2 +
```

```
                beta[25]*FVC[i]*cursmoke[i] +
                beta[26]*FVC[i]*packyrs[pid[i],survey[i]] +
                beta[27]*FVC[i]*SESmid[pid[i]] + beta[28]*FVC[i]*SESlow[pid[i]] +
                beta[29]*FVC[i]*disadv[i] + beta[30]*FVC[i]*asthma[i] +

                u1[pid[i],1]*FEV1[i] + u1[pid[i],2]*FEV1[i]*age[i] +
                u1[pid[i],3]*FVC[i] + u1[pid[i],4]*FVC[i]*age[i]

    Y[i] ~ dnorm(mu[i], tau.res*weight[i])
  }

  # Residual variance
  tau.res <- pow(sigma.res,-2)
  sigma.res ~ dunif(0,1000)

  # Priors:
  # Fixed intercept and slope
  for (b in 1:30) {
    beta[b] ~ dnorm(0.0,1.0E-5)
  }


# Imputation models for missing covariates

  for(i in 1:N ) {
    # Smoking
    cursmoke[i] ~ dbern(theta.smoke)
    asthma[i] ~ dbern(theta.asthma)
  }

  for (j in 1:K) {
    # SES
    SES[j] ~ dcat(p.SES)
    SESmid[j] <- equals(SES[j], 2)
    SESlow[j] <- equals(SES[j], 3)

    for (t in 1:3) {
      pyb[j,t] ~ dgamma(gaj[j, t] , gbj[j, t])
      gaj[j, t] <- ga[t] * smoked[j, t] + 0.0001
      gbj[j, t] <- gb[t] * smoked[j, t] + 0.0001
      smoked[j, t] ~ dbern(pSmk[t])
    }
    packyrs[j,1] <- pyb[j,1]
    packyrs[j,2] <- pyb[j,2] + packyrs[j,1]
    packyrs[j,3] <- pyb[j,3] + packyrs[j,2]
    everSmoked[j,1] <- packyrs[j,1]>0
    everSmoked[j,2] <- packyrs[j,2]>0
    everSmoked[j,3] <- packyrs[j,3]>0
  }

  for (t in 1:3) {
    ga[t] <- gmean[t]^2/gsd[t]^2
    gb[t] <- gmean[t]/gsd[t]^2
    logit(pSmk[t]) <- bSmk[t]
    bSmk[t] ~ dnorm(0.0,1.0E-5)
  }

  # Priors for hyperparameters
  theta.smoke ~ dbeta(0.5,0.5)
  theta.asthma ~ dbeta(0.5,0.5)
  p.SES[1:3] ~ ddirch(noninf[1:3])

}
```

Supplemental Table 1: Ten most frequent occupations by exposure category, across both cohorts (ECRHS and SAPALDIA), with number and percentage of exposed participants.

Biological dust

ISCO code	Occupation	N (%)
9132	Helpers and cleaners in offices, hotels and other establishments	426 (7.0%)
5132	Institution-based personal care workers	413 (6.8%)
2230	Nursing and midwifery professionals	360 (5.9%)
3231	Nursing associate professionals	355 (5.8%)
9141	Building caretakers	289 (4.7%)
5122	Cooks	228 (3.7%)
2221	Medical doctors	179 (2.9%)
5133	Home-based personal care workers	167 (2.7%)
9131	Domestic helpers and cleaners	162 (2.6%)
9130	Domestic and related helpers, cleaners and launderers	156 (2.6%)

Mineral Dust

ISCO code	Occupation	N (%)
9132	Helpers and cleaners in offices, hotels and other establishments	426 (8.5%)
9141	Building caretakers	289 (5.8%)
7231	Motor vehicle mechanics and fitters	171 (3.4%)
9131	Domestic helpers and cleaners	162 (3.2%)
9130	Domestic and related helpers, cleaners and launderers	156 (3.1%)
3471	Decorators and commercial designers	154 (3.1%)
8324	Heavy truck and lorry drivers	153 (3.1%)
7137	Building and related electricians	152 (3.0%)
7136	Plumbers and pipe fitters	142 (2.8%)
6130	Market-oriented crop and animal producers	129 (2.6%)

Gases & fumes

ISCO code	Occupation	N (%)
9132	Helpers and cleaners in offices, hotels and other establishments	426 (4.6%)
5132	Institution-based personal care workers	413 (4.4%)
2230	Nursing and midwifery professionals	360 (3.9%)
9141	Building caretakers	289 (3.1%)
5123	Waiters, waitresses and bartenders	284 (3.1%)
5122	Cooks	228 (2.4%)
3340	Other teaching associate professionals	191 (2.1%)
7231	Motor vehicle mechanics and fitters	171 (1.8%)
5133	Home-based personal care workers	167 (1.8%)
9131	Domestic helpers and cleaners	162 (1.7%)

Vapors, Gases, Dusts & Fumes

ISCO code	Occupation	N (%)
9132	Helpers and cleaners in offices, hotels and other establishments	426 (4.0%)
5132	Institution-based personal care workers	413 (3.9%)
2230	Nursing and midwifery professionals	360 (3.4%)
3231	Nursing associate professionals	355 (3.3%)
9141	Building caretakers	289 (2.7%)
5123	Waiters, waitresses and bartenders	284 (2.7%)
5122	Cooks	228 (2.1%)
3340	Other teaching associate professionals	191 (1.8%)
2221	Medical doctors	179 (1.7%)
7231	Motor vehicle mechanics and fitters	171 (1.6%)

Herbicides

ISCO code	Occupation	N (%)
6130	Market-oriented crop and animal producers	129 (27.7%)
6112	Tree and shrub crop growers	102 (21.9%)
6113	Gardeners, horticultural and nursery growers	82 (17.6%)
6111	Field crop and vegetable growers	61 (13.1%)
6141	Forestry workers and loggers	23 (4.9%)
9211	Farm-hands and labourers	16 (3.4%)
6114	Mixed-crop growers	13 (2.8%)
8331	Motorised farm and forestry plant operators	12 (2.6%)
3212	Agronomy and forestry technicians	10 (2.1%)
6100	Market-oriented skilled agricultural and fishery workers	9 (1.9%)

Insecticides

ISCO code	Occupation	N (%)
6130	Market-oriented crop and animal producers	129 (22.3%)
6112	Tree and shrub crop growers	102 (17.6%)
6113	Gardeners, horticultural and nursery growers	82 (14.2%)
9333	Freight handlers	63 (10.9%)
6111	Field crop and vegetable growers	61 (10.5%)
6121	Dairy and livestock producers	30 (5.2%)
6129	Market-oriented animal producers and related workers not elsewhere classified	20 (3.5%)
9211	Farm-hands and labourers	16 (2.8%)
6114	Mixed-crop growers	13 (2.2%)
2223	Veterinarians	12 (2.1%)

Fungicides

ISCO code	Occupation	N (%)
6130	Market-oriented crop and animal producers	129 (20.2%)
6112	Tree and shrub crop growers	102 (15.9%)
8211	Machine-tool operators	100 (15.6%)

ISCO code	Occupation	N (%)
6113	Gardeners, horticultural and nursery growers	82 (12.8%)
6111	Field crop and vegetable growers	61 (9.5%)
6121	Dairy and livestock producers	30 (4.7%)
6129	Market-oriented animal producers and related workers not elsewhere classified	20 (3.1%)
8141	Wood-processing-plant operators	18 (2.8%)
9211	Farm-hands and labourers	16 (2.5%)
6114	Mixed-crop growers	13 (2.0%)

All pesticides

ISCO code	Occupation	N (%)
6130	Market-oriented crop and animal producers	129 (17.2%)
6112	Tree and shrub crop growers	102 (13.6%)
8211	Machine-tool operators	100 (13.3%)
6113	Gardeners, horticultural and nursery growers	82 (10.9%)
9333	Freight handlers	63 (8.4%)
6111	Field crop and vegetable growers	61 (8.1%)
6121	Dairy and livestock producers	30 (4.0%)
6141	Forestry workers and loggers	23 (3.1%)
6129	Market-oriented animal producers and related workers not elsewhere classified	20 (2.7%)
8141	Wood-processing-plant operators	18 (2.4%)

Aromatic solvents

ISCO code	Occupation	N (%)
7231	Motor vehicle mechanics and fitters	171 (5.8%)
3471	Decorators and commercial designers	154 (5.2%)
7136	Plumbers and pipe fitters	142 (4.8%)
6130	Market-oriented crop and animal producers	129 (4.4%)
7241	Electrical mechanics and fitters	125 (4.2%)
7124	Carpenters and joiners	123 (4.2%)
6112	Tree and shrub crop growers	102 (3.5%)
3211	Life science technicians	95 (3.2%)
7233	Agricultural- or industrial-machinery mechanics and fitters	92 (3.1%)
7222	Tool-makers and related workers	88 (3.0%)

Chlorinated solvents

ISCO code	Occupation	N (%)
7231	Motor vehicle mechanics and fitters	171 (7.5%)
3471	Decorators and commercial designers	154 (6.7%)
5141	Hairdressers, barbers, beauticians and related workers	145 (6.3%)
7136	Plumbers and pipe fitters	142 (6.2%)
7241	Electrical mechanics and fitters	125 (5.5%)
7233	Agricultural- or industrial-machinery mechanics and fitters	92 (4.0%)
7222	Tool-makers and related workers	88 (3.9%)

ISCO code	Occupation	N (%)
3111	Chemical and physical science technicians	87 (3.8%)
7422	Cabinet makers and related workers	87 (3.8%)
7141	Painters and related workers	83 (3.6%)

Other solvents

ISCO code	Occupation	N (%)
5132	Institution-based personal care workers	413 (8.1%)
2230	Nursing and midwifery professionals	360 (7.0%)
3231	Nursing associate professionals	355 (6.9%)
2221	Medical doctors	179 (3.5%)
7231	Motor vehicle mechanics and fitters	171 (3.3%)
5133	Home-based personal care workers	167 (3.3%)
3471	Decorators and commercial designers	154 (3.0%)
5141	Hairdressers, barbers, beauticians and related workers	145 (2.8%)
7136	Plumbers and pipe fitters	142 (2.8%)
5130	Personal care and related workers	126 (2.5%)

Metals

ISCO code	Occupation	N (%)
7231	Motor vehicle mechanics and fitters	171 (8.0%)
7136	Plumbers and pipe fitters	142 (6.6%)
7241	Electrical mechanics and fitters	125 (5.8%)
8211	Machine-tool operators	100 (4.7%)
7233	Agricultural- or industrial-machinery mechanics and fitters	92 (4.3%)
7222	Tool-makers and related workers	88 (4.1%)
3114	Electronics and telecommunications engineering technicians	87 (4.1%)
7141	Painters and related workers	83 (3.9%)
2144	Electronics and telecommunications engineers	76 (3.6%)
2145	Mechanical engineers	76 (3.6%)

Supplemental Table 2: Effect of occupational exposures on lung function decline after 25 intensity-years of exposure, compared to unexposed to the respective agent, overall and stratified by sex and smoking status (ever / never smokers), separately for the two participating cohorts (ECRHS and SAPALDIA)

(a) ECRHS

	FEV1		FVC		FEV1FVC	
Biological dust	-9.76 (-28.22 – 7.83)		11.72 (-10.27 – 32.62)		**-0.62 (-1.05 – -0.19)**	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.49 (-1.27 – 0.30)	-0.46 (-1.38 – 0.48)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	**-0.71 (-1.29 – -0.13)**	-0.68 (-1.56 – 0.21)
Mineral Dust	-9.68 (-23.90 – 5.10)		**22.41 (5.17 – 39.73)**		**-0.88 (-1.22 – -0.53)**	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.57 (-1.23 – 0.11)	0.09 (-1.05 – 1.23)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	**-1.10 (-1.52 – -0.69)**	-0.45 (-1.57 – 0.66)
Gases & fumes	-2.48 (-15.51 – 10.75)		**24.43 (9.30 – 39.56)**		**-0.60 (-0.90 – -0.28)**	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.27 (-0.83 – 0.28)	-0.25 (-1.12 – 0.64)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	**-0.75 (-1.14 – -0.36)**	-0.72 (-1.54 – 0.08)
Vapors, Gases, Dusts & Fumes	-2.58 (-12.75 – 8.01)		**23.72 (11.43 – 36.07)**		**-0.63 (-0.88 – -0.39)**	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.35 (-0.80 – 0.11)	-0.21 (-0.89 – 0.49)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	**-0.79 (-1.09 – -0.48)**	-0.65 (-1.27 – 0.02)
Herbicides	**-68.51 (-120.61 – -13.48)**		**-73.44 (-136.76 – -9.54)**		-0.58 (-1.84 – 0.72)	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.46 (-3.20 – 2.40)	1.31 (-2.22 – 4.97)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	-1.06 (-2.55 – 0.43)	0.69 (-2.34 – 3.79)
Insecticides	**-39.07 (-75.81 – -2.82)**		-24.81 (-69.02 – 18.45)		-0.72 (-1.58 – 0.13)	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	0.33 (-1.38 – 2.18)	1.13 (-1.27 – 3.59)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	**-1.31 (-2.37 – -0.22)**	-0.55 (-2.56 – 1.48)
Fungicides	**-40.48 (-77.31 – -3.47)**		-30.32 (-75.21 – 14.96)		-0.60 (-1.48 – 0.28)	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	0.25 (-1.60 – 2.26)	1.49 (-1.09 – 4.10)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	**-1.17 (-2.21 – -0.11)**	0.01 (-2.03 – 2.09)
All pesticides	**-44.45 (-79.01 – -9.33)**		-29.94 (-69.64 – 11.85)		-0.76 (-1.57 – 0.07)	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	0.30 (-1.42 – 2.03)	1.01 (-1.24 – 3.43)

	FEV1		FVC		FEV1FVC	
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	**-1.27 (-2.25 – -0.26)**	-0.56 (-2.50 – 1.47)
Aromatic solvents	14.20 (-13.96 – 42.54)		**38.00 (3.67 – 71.52)**		-0.48 (-1.19 – 0.22)	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.44 (-1.67 – 0.83)	-0.00 (-2.28 – 2.29)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	-0.58 (-1.48 – 0.29)	-0.16 (-2.28 – 1.95)
Chlorinated solvents	-12.04 (-33.56 – 9.22)		4.85 (-20.73 – 29.91)		-0.51 (-1.04 – 0.04)	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.45 (-1.44 – 0.51)	-0.17 (-2.50 – 2.35)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	-0.55 (-1.18 – 0.06)	-0.25 (-2.43 – 2.09)
Other solvents	7.47 (-14.80 – 29.29)		22.81 (-3.15 – 48.32)		-0.39 (-0.92 – 0.14)	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.66 (-1.70 – 0.37)	-0.57 (-1.64 – 0.60)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	-0.36 (-1.13 – 0.43)	-0.24 (-1.11 – 0.62)
Metals	-12.87 (-32.03 – 6.81)		12.14 (-11.49 – 35.27)		**-0.70 (-1.18 – -0.23)**	
Never smokers, men / women	1.27 (-4.40 – 7.33)	-4.91 (-14.65 – 4.71)	4.85 (-0.98 – 10.35)	-2.82 (-11.49 – 5.98)	-0.43 (-1.29 – 0.44)	0.94 (-1.34 – 3.32)
Ever smokers, men / women	-1.52 (-5.73 – 2.56)	-7.74 (-16.88 – 1.23)	**6.33 (2.55 – 10.09)**	-1.35 (-9.53 – 6.98)	**-0.92 (-1.48 – -0.36)**	0.45 (-1.76 – 2.77)

Results are posterior medians and 95% CrI in parentheses. Numbers represent absolute change in ml for FEV1 and FVC, and relative %change for FEV1/FVC. A negative sign indicates a lower value compared to fully unexposed to the respective agent. Adjusted for age, sex, current smoking, cumulative smoking pack-years, socioeconomic status and early life disadvantage score. All lung function measurements are without bronchodilation.

(b) SAPALDIA

	FEV1		FVC		FEV1FVC	
Biological dust	**-20.33 (-39.79 – -1.15)**		15.28 (-5.70 – 35.96)		**-1.04 (-1.56 – -0.52)**	
Never smokers, men / women	-25.61 (-53.97 – 4.43)	5.27 (-32.33 – 44.40)	5.48 (-25.42 – 36.66)	**66.72 (26.09 – 108.18)**	-0.70 (-1.48 – 0.08)	**-1.36 (-2.43 – -0.32)**
Ever smokers, men / women	**-34.82 (-64.82 – -3.57)**	-3.47 (-47.72 – 39.11)	-13.37 (-45.83 – 19.90)	48.00 (-0.24 – 94.72)	**-1.05 (-1.92 – -0.19)**	**-1.71 (-2.88 – -0.56)**
Mineral Dust	-12.45 (-30.54 – 5.27)		10.55 (-9.43 – 30.02)		**-0.57 (-1.06 – -0.07)**	
Never smokers, men / women	-11.52 (-40.80 – 17.45)	11.72 (-29.79 – 51.91)	14.26 (-18.31 – 45.85)	33.85 (-12.26 – 78.95)	-0.45 (-1.25 – 0.37)	-0.41 (-1.56 – 0.74)
Ever smokers, men / women	-22.18 (-46.39 – 2.37)	0.82 (-44.05 – 45.43)	-0.89 (-27.38 – 26.32)	19.16 (-29.29 – 67.30)	**-0.68 (-1.35 – -0.01)**	-0.64 (-1.86 – 0.61)
Gases & fumes	-5.81 (-22.33 – 10.62)		**22.59 (4.13 – 40.68)**		**-0.64 (-1.08 – -0.19)**	
Never smokers, men / women	-13.35 (-40.04 – 11.98)	-2.53 (-48.16 – 42.93)	19.57 (-8.49 – 47.04)	**58.72 (10.18 – 108.28)**	-0.60 (-1.32 – 0.13)	-1.25 (-2.51 – 0.00)
Ever smokers, men / women	-3.64 (-25.43 – 18.23)	7.53 (-36.29 – 51.53)	14.07 (-9.51 – 37.74)	**53.60 (6.39 – 102.21)**	-0.50 (-1.10 – 0.10)	-1.14 (-2.35 – 0.08)

	FEV1		FVC		FEV1FVC	
Vapors, Gases, Dusts & Fumes	-9.58 (-21.87 – 3.01)		**18.02 (4.44 – 31.96)**		**-0.67 (-1.01 – -0.32)**	
Never smokers, men / women	-17.12 (-36.41 – 2.13)	-5.57 (-35.59 – 23.53)	12.67 (-8.75 – 33.19)	**43.10 (10.98 – 75.71)**	**-0.60 (-1.11 – -0.07)**	**-1.15 (-1.96 – -0.33)**
Ever smokers, men / women	-6.73 (-23.78 – 10.54)	4.86 (-26.17 – 34.98)	12.23 (-6.54 – 31.17)	**42.94 (9.10 – 76.75)**	**-0.53 (-1.00 – -0.08)**	**-1.08 (-1.94 – -0.23)**
Herbicides	5.65 (-36.36 – 47.10)		14.23 (-31.26 – 58.31)		-0.14 (-1.31 – 1.02)	
Never smokers, men / women	-9.95 (-72.21 – 57.68)	31.91 (-51.93 – 117.07)	4.04 (-64.10 – 73.82)	51.94 (-39.70 – 143.97)	-0.35 (-2.11 – 1.42)	0.21 (-2.14 – 2.56)
Ever smokers, men / women	-5.16 (-71.98 – 63.89)	36.54 (-49.93 – 119.46)	-5.78 (-80.47 – 66.90)	41.19 (-51.62 – 132.75)	-0.26 (-2.16 – 1.71)	0.27 (-2.04 – 2.70)
Insecticides	1.01 (-25.85 – 27.58)		5.73 (-24.17 – 34.81)		-0.07 (-0.79 – 0.68)	
Never smokers, men / women	-2.94 (-41.46 – 38.06)	17.76 (-30.20 – 63.96)	4.43 (-38.15 – 48.13)	32.06 (-19.92 – 83.38)	-0.12 (-1.18 – 0.97)	0.01 (-1.30 – 1.34)
Ever smokers, men / women	-13.13 (-61.58 – 37.11)	7.36 (-50.67 – 65.00)	-21.87 (-74.58 – 31.50)	5.99 (-57.71 – 68.63)	-0.07 (-1.42 – 1.30)	0.05 (-1.57 – 1.71)
Fungicides	4.34 (-36.26 – 43.67)		13.95 (-29.23 – 56.33)		-0.18 (-1.34 – 0.92)	
Never smokers, men / women	-13.56 (-76.90 – 49.60)	28.09 (-54.83 – 110.07)	0.39 (-68.34 – 68.18)	48.77 (-42.41 – 139.81)	-0.36 (-2.07 – 1.33)	0.15 (-2.15 – 2.48)
Ever smokers, men / women	-5.43 (-69.28 – 55.62)	34.80 (-42.94 – 116.26)	-4.59 (-72.76 – 63.91)	44.28 (-40.55 – 130.40)	-0.33 (-2.13 – 1.48)	0.20 (-2.02 – 2.48)
All pesticides	0.53 (-26.06 – 27.38)		6.45 (-22.99 – 36.02)		-0.08 (-0.82 – 0.64)	
Never smokers, men / women	-3.68 (-42.31 – 34.60)	18.11 (-29.77 – 66.47)	3.96 (-39.31 – 46.27)	31.71 (-19.57 – 84.72)	-0.14 (-1.20 – 0.94)	0.07 (-1.21 – 1.39)
Ever smokers, men / women	-13.46 (-62.36 – 33.92)	8.07 (-49.25 – 65.47)	-18.34 (-70.69 – 33.74)	9.95 (-54.47 – 71.99)	-0.18 (-1.52 – 1.19)	0.02 (-1.57 – 1.64)
Aromatic solvents	-14.66 (-49.14 – 19.40)		5.11 (-31.27 – 41.31)		-0.46 (-1.37 – 0.50)	
Never smokers, men / women	-42.72 (-94.34 – 9.07)	28.85 (-76.78 – 131.54)	-27.33 (-85.58 – 28.83)	74.84 (-33.79 – 185.12)	-0.46 (-1.84 – 1.01)	-0.50 (-3.27 – 2.44)
Ever smokers, men / women	-7.81 (-53.28 – 37.77)	64.70 (-35.33 – 163.51)	8.68 (-41.38 – 57.93)	**111.39 (4.27 – 217.92)**	-0.45 (-1.72 – 0.87)	-0.51 (-3.16 – 2.32)
Chlorinated solvents	-12.40 (-37.69 – 12.63)		-4.38 (-32.08 – 22.70)		-0.27 (-0.98 – 0.43)	
Never smokers, men / women	-12.50 (-55.02 – 30.36)	-41.86 (-130.82 – 46.18)	-12.46 (-60.29 – 33.73)	-28.22 (-125.07 – 72.43)	0.04 (-1.16 – 1.25)	-0.43 (-2.91 – 2.08)
Ever smokers, men / women	-8.32 (-40.35 – 24.35)	-38.49 (-128.73 – 54.74)	2.29 (-31.81 – 36.44)	-13.14 (-110.89 – 87.01)	-0.38 (-1.27 – 0.52)	-0.88 (-3.38 – 1.68)
Other solvents	-24.04 (-49.73 – 1.11)		17.83 (-10.08 – 45.36)		**-0.99 (-1.69 – -0.29)**	
Never smokers, men / women	-36.95 (-82.40 – 7.04)	-36.36 (-85.03 – 12.21)	30.10 (-18.73 – 80.38)	16.65 (-35.12 – 69.27)	**-1.38 (-2.59 – -0.13)**	-1.24 (-2.55 – 0.12)
Ever smokers, men / women	-14.64 (-55.94 – 27.47)	-14.06 (-53.77 – 28.00)	21.99 (-24.13 – 67.10)	8.00 (-35.61 – 53.53)	-0.84 (-1.95 – 0.30)	-0.70 (-1.78 – 0.39)
Metals	-18.74 (-42.78 – 5.34)		-3.28 (-28.85 – 22.32)		-0.46 (-1.14 – 0.19)	
Never smokers, men / women	-27.93 (-69.89 – 13.38)	**-147.12 (-281.53 – -8.72)**	-18.45 (-63.93 – 27.62)	-88.76 (-238.04 – 54.05)	-0.24 (-1.36 – 0.91)	-3.28 (-7.04 – 0.75)
Ever smokers, men / women	-8.22 (-37.98 – 20.44)	-128.01 (-254.97 – 5.40)	8.09 (-24.69 – 38.98)	-62.51 (-206.22 – 74.48)	-0.43 (-1.24 – 0.41)	-3.44 (-7.18 – 0.27)

Results are posterior medians and 95% CrI in parentheses. Numbers represent absolute change in ml for FEV1 and FVC, and relative

%change for FEV1/FVC. A negative sign indicates a lower value compared to fully unexposed to the respective agent. Adjusted for age, sex, current smoking, cumulative smoking pack-years, socioeconomic status and early life disadvantage score. All lung function measurements are without bronchodilation.

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